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Chromosome Abnormalities In Blastocysts Of The Rabbit Following Delayed Fertilization

Evelyn Louise Shaver

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CHROMOSOME ABNORMALITIES IN BLASTOCYSTS OF THE
RABBIT FOLLOWING DELAYED FERTILIZATION

by

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Department of Anatomy

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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In addition, I am indebted to each of my co-workers for the continuous offering of their talents. The majority of the hundreds of karyotypes were printed and assembled by Mrs. G. Feleki and Miss Patricia Carter. Mr. K. McGill and his staff provided the care for the animals during the past three years.

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ABSTRACT

A study was made of the chromosome complement of blastocysts recovered from rabbits in which delayed fertilization had occurred. Delayed fertilization was accomplished by an injection of 25 I. U. chorionic gonadotrophin followed by insemination at various intervals up to 14 hours after injection. The time at which ovulation took place was assumed to be 10 hours after gonadotrophin injection.

As the interval between injection and mating increased, the number of blastocysts recovered six days later decreased. The greatest decline in recovery of blastocysts occurred when mating was delayed at least 10 hours after gonadotrophin injection. Also, a greater proportion of small blastocysts, which appeared to be retarded in development, was recovered from animals mated later than 10 hours after injection of gonadotrophin.

A 13% incidence of triploidy was found in blastocysts from rabbits mated six to nine hours after injection. Ten triploids were found among 80 blastocysts examined chromosomally at these intervals. Seven of the triploids had an XXY sex chromosome complex and three triploids an XXX sex chromosome complex. Triploidy was not found at other delay intervals.

Three chromosome abnormalities, a mixoploid and two 43/44 chromosome mosaics, were found among 57 blastocysts from rabbits mated two to four hours after gonadotrophin injection. In addition, a 44/47 chromosome mosaic was found in a blastocyst from a rabbit mated 13 hours after injection. The incidence of chromosome abnormalities at these intervals was approximately five percent and was not significantly different from the seven percent of chromosome aberrations found in blastocysts from animals mated immediately after injection of gonadotrophin. A pentaploid, two mixoploids, a trisomy and a blastocyst with 44 chromosomes but an abnormal karyotype were found among 73 blastocysts from rabbits in which mating was not delayed.

Fifty-two blastocysts from a control group of rabbits which received no gonadotrophin prior to mating resulted in the finding of only one chromosome abnormality, a 44/45 chromosome mosaic.

When doses of chorionic gonadotrophin varying from 50 I.U. to 300 I.U. were administered immediately prior to mating, a decrease in the number of blastocysts recovered occurred with doses of 200 I.U. and 300 I.U. Also, two 43/44 chromosome mosaics were found among 28 blastocysts from rabbits injected with 50 I.U. or 75 I.U. gonadotrophin. Thus, a slight increase in the number of chromosome abnormalities was found in blastocysts from animals injected with gonadotrophin immediately prior to mating, compared with blastocysts from rabbits receiving no hormone.

The sex chromosome complex was XY or male in 54% and female or XX in 46% of the blastocysts. No difference in the sex ratio was noted with delayed mating.

A delay of six to nine hours between gonadotrophin injection and mating was found to increase significantly the number of triploid blastocysts. As triploidy has been reported to be the second most common chromosome aberration in human spontaneous abortions, delayed fertilization may be an important causal factor.

I. INTRODUCTION

The investigation by Carr (1963, 1965), in which chromosome abnormalities were found in 22% of early spontaneous abortions, demonstrated the importance of chromosome aberrations in embryonic and foetal loss in the human. A wide range of chromosome abnormalities was found, including complete extra sets of chromosomes giving triploid and tetraploid karyotypes; one extra chromosome in the complement, resulting in various trisomies, and specimens with an XO sex chromosome complex. The finding of an increased incidence of chromosome abnormalities in early spontaneous abortions has been confirmed by a number of investigators (Standardization of procedures for chromosome studies in abortion, 1966).

In 1963, Witschi and Laguens studied the chromosomes of amphibian embryos which had developed from ova retained in the uterus up to five days before fertilization took place. A significant proportion of the embryos from the overripe ova were found to have chromosome abnormalities consisting of polyploid, as well as trisomic and monosomic chromosome complements. It was concluded by Witschi and Laguens that overripeness of the egg was a major cause of chromosome non-disjunction in meiotic as well as mitotic divisions.

Delayed fertilization of ova has been observed in laboratory mammals to result in a decreased litter size at birth, anomalies in early developmental stages and increased embryonic mortality in guinea pigs (Blandau and Young, 1939), rats (Blandau and Jordan, 1941) and rabbits (Hammond, 1934; Chang, 1952a). However, no investigations had been reported of chromosome studies in mammalian embryos or foetuses following delayed fertilization. Thus, a study was undertaken to determine the effect of delayed fertilization on the number and types of chromosome abnormalities in preimplantation blastocysts of a mammal. The rabbit was chosen as the experimental animal because of the precise time at which ovulation occurs.

II. HISTORICAL REVIEW

A. The Chromosomes of the Domestic Rabbit, *Oryctolagus cuniculus* (L.)

The diploid number of chromosomes for the domestic rabbit, *Oryctolagus cuniculus* (L.), formerly known as *Lepus cuniculus*, was first established by Painter (1926). A somatic count of 44 was found in amnion cells from 14-day embryos. Extending the study to include spermatogenesis in the adult male, Painter (1926) found a diploid number of 44 in spermatogonia and a haploid number of 22 chromosomes in primary spermatocytes. No difference in the diploid number was noted when various races within the species were studied.

Confirmation of the diploid count for the rabbit came from a number of investigators and references to them are given by Makino (1951). With the advent of modern tissue culture and chromosome techniques, no change in the somatic number of chromosomes was found. Melander (1956) culturing embryonic lung cells, Sarkar, Basu and Miller (1962) using cornea, and Ray and Williams (1966) examining cells from leucocyte cultures, confirmed the diploid count to be 44 chromosomes in the domestic rabbit.

The first complete analysis of the chromosome complement of the rabbit according to length and centromere position was made by Melander (1956). The chromosomes were arranged in pairs according to length from camera lucida tracings of metaphase plates. The X-chromosome was thought to be a medium length chromosome and the Y-chromosome to be one of the smallest chromosomes of the complement.

Since Melander's publication, at least seven papers have appeared describing the karyotype of the rabbit. Unfortunately, no definite classification has been organized for animal chromosomes as was accomplished by the Denver Conference for the karyotype of the human (International Study Group, 1960). As a result, in the karyotype of the rabbit, an individual chromosome pair may be given a different number and be placed in a different group in each of the published reports. Two basic classifications, however, have developed; the first depending mainly on chromosome length and the second classification based on the position of the centromere.

The idiogram published by Nichols, Levan, Hansen-Melander and Melander (1965) forms the basis for chromosome classification used in the present study of rabbit blastocysts and therefore will be described in greater detail than the other published karyotypes. Nichols et al (1965) derived arm ratios (length of the long arm/length of the short arm) for the chromosomes and grouped them according to the specifications and nomenclature proposed by Levan, Fredga and Sandberg (1964).

The chromosomes were divided into four groups according to centromere position and arm ratio, then arranged in diminishing order of size within each group. Figures 1 and 2 are karyotypes grouped according to this classification. The sex chromosome complex is XY or male in Figure 1, and XX or female in Figure 2. The four different groups are formed as follows:

Group m: Chromosomes 1-6. Centromeres median to submedian; arm ratio 1.0-1.7. Three pairs of large, one pair of medium and two pairs of small sized chromosomes.

Group sm: Chromosomes 7-12. Centromeres submedian; arm ratio 1.7-3.0. One pair of large, three pairs of medium and two pairs of small chromosomes. The X-chromosome is one of the medium sized chromosomes and is designated as number 8, although at times it is indistinguishable from pairs 9 and 10.

Group st: Chromosomes 13-18. Centromeres subterminal; arm ratio 3.0-7.0. Six pairs of chromosomes of large to medium size.

Group t: Chromosomes 19-22. Centromeres subterminal to terminal; arm ratio 7.0- ∞ . Two pairs of small and two pairs of very small chromosomes.

The Y-chromosome is the smallest chromosome of the complement. Nichols et al (1965) placed it in the sm group as it appeared to have a submedian centromere. In the karyotypes in this thesis, the Y-chromosome is placed at the end of the t group as it is often difficult

to distinguish it from chromosome pairs 21 and 22.

The numbers given to the 21 pairs of autosomes by various authors are compared in Table I. The X-chromosome, which is listed as number 8 by Nichols et al (1965), is not numbered in the other karyotypes.

Length of the chromosome was taken as the main feature for numbering the chromosomes by Melander (1956), Teplitz and Ohno (1965), McMichael, Wagner, Nowell and Hungerford (1963), Pruniéras, Jacquemont and Mathivon (1965) and Ray and Williams (1966). Neither Melander (1956) nor Teplitz and Ohno (1963) grouped the chromosomes; the autosomal pairs were numbered and arranged in diminishing size in a single line.

The classification proposed by McMichael et al (1963) and followed by Pruniéras et al (1965) arranged the autosomes into six groups as follows:

Group 1. Chromosomes 1-4; large chromosomes with median and submedian centromeres.

Group 2. Chromosomes 5-8; large chromosomes with subterminal centromeres.

Group 3. Chromosomes 9-11; medium length chromosomes with subterminal centromeres.

Group 4. Chromosomes 12-17; medium length chromosomes with submedian centromeres and small chromosomes with median centromeres.

Group 5. Chromosomes 18 and 19; medium size chromosomes with subterminal to terminal centromeres.

Group 6. Chromosomes 20 and 21; small chromosomes with subterminal to terminal centromeres.

Ray and Williams (1966) used a modification of the classification given by McMichael et al (1963). The chromosomes were arranged in seven instead of six groups, lettered A-G. The extra group was formed by taking the smallest two pairs of median centromere chromosomes from Group 4 and placing them in a separate group between Groups 5 and 6.

The second major classification used to group rabbit chromosomes was based primarily on centromere position and was followed by Nichols et al (1965), Sarkar et al (1962) and Clausen and Syverton (1962). The idiogram published by Nichols et al (1965) has been described previously. Sarkar et al (1962) arranged the autosomes into nine pairs of chromosomes with median or slightly submedian centromeres, four pairs of medium length chromosomes with submedian centromeres, four pairs of large chromosomes with subterminal centromeres and four pairs of smaller chromosomes with almost terminal centromeres. Clausen and Syverton (1962) also divided the chromosome complement into four groups. Unfortunately, the published karyotype was of poor quality and it was difficult to determine the chromosomes corresponding to the Nichols et al (1965) karyotype. In addition, Clausen and Syverton

(1962) reported finding only three pairs of small chromosomes with almost terminal centromeres. All the other authors have stated that there are four pairs of these chromosomes.

Some degree of confusion regarding the morphology of the X-chromosome is apparent from reading the papers published on the chromosomes of the rabbit. The main point of agreement by the various authors is that the X-chromosome is one of the medium-sized chromosomes (Painter, 1926; Melander, 1956; Sarkar et al, 1962; McMichael et al, 1963; Pruniéras et al, 1965; Nichols et al, 1965; Ray and Williams, 1966). The one exception was Clausen and Syvertson (1962); they thought the X-chromosome was a small submedian chromosome. The position of the centromere, however, has been reported to be median, submedian, subterminal and almost terminal. In fact, Pruniéras et al (1965) observed two different forms of the X-chromosome. At times, they thought the centromere was median in location and at other times slightly submedian. Teplitz and Ohno (1963) declared that the X-chromosome was a middle-sized chromosome with a terminal centromere. Ray and Williams (1966) with the aid of autoradiographic studies, in which the cells in culture were labelled with tritiated thymidine, determined the late replicating chromosomes. In other studies, these chromosomes have been the sex chromosomes. Therefore, Ray and Williams (1966) decided that the X-chromosome

was a medium-sized chromosome with a subterminal centromere. However, from their published karyotype it would appear that the chromosome designated as the X-chromosome has slightly longer short arms than the other chromosomes with subterminal centromeres and therefore, is more submedian in centromere position. Nichols et al (1965) described the X-chromosome as belonging to a group of chromosomes with submedian centromeres. This was also the opinion of Sarkar et al (1962).

The sex chromosome complex of metaphase plates may be readily determined, however, by a count of the number of very small chromosomes. The Y-chromosome in the rabbit complement has been well documented as a small chromosome at times indistinguishable from autosome pairs 21 and 22 (Fig. 1). Therefore, if five very small chromosomes are present, the sex chromosome complex is XY or male, whereas it is female or XX if only four very small chromosomes are in the metaphase plate.

Certain chromosomes of the rabbit complement have been reported to bear satellites. Satellites are a form of secondary constriction located at the ends of the short arms of some chromosomes. The satellites stain, at times, and seem to be attached to the short arms of the chromosome by two fine threads. Pruniéras et al (1965) and Nichols et al (1965) observed satellites to be present frequently on the first pair of very small chromosomes with nearly terminal centromeres (pair No. 21). It was suggested by Ray and Williams (1966) that both pairs

of very small autosomes at times bore satellites. Pruni  ras et al (1965) reported satellites to be present, also, on chromosome pairs numbered 5 or 6 or 7, 10, and 20. Transposed to the numbering system of Nichols et al (1965) this would be chromosome pairs 13 or 14 or 15, 17 and 21 (Table 1). The karyotype shown in Figure 1 depicts satellites on two of these pairs of chromosomes (numbers 13 and 17). Also, a secondary constriction, appearing as a pale zone, may be seen in chromosomes 5 and 11.

B. Abnormalities in Mammalian Embryos Associated with Delayed Fertilization

Sterility and decreased fertility have often been associated with delayed fertilization. A reduction in litter size and lowered fertility was noted in the ewe (Quinlan and Mare  , 1931) and in the rabbit (Biedl, Peters and Hofst  tter, 1921; Hammond and Marshall, 1925) when mating was delayed. When coitus occurred shortly after heat and ovulation in the sow (Lewis, 1911) and late in the oestrous cycle in the monkey (Hartman, 1932) and chimpanzee (Elder, 1938), sterility invariably arose. These early studies, however, did not determine whether lowered fertility was due to ova not being fertilized or to ova not developing normally through the early cleavage stages, leading to eventual embryonic or foetal death.

Evidence later accumulated which demonstrated clearly that the period of ovum viability was short, and that the percentage of ova fertilized decreased in the rabbit (Hammond, 1934), ferret (Hammond and

Walton, 1934), guinea pig (Blandau and Young, 1939) and rat (Blandau and Jordan, 1941), as the time between ovulation and fertilization increased. In all animals studied, regardless of whether an oestrous cycle was present and thus ovulation was spontaneous, or the animal ovulated only after coitus, such as the rabbit, cat and ferret, delayed fertilization resulted in the percentage of ova fertilized declining rapidly with time. For example, in the golden hamster, which ovulates about eight hours after the onset of oestrus, only three to nine percent of ova were fertilized when fertilization was delayed until nine to twelve hours after ovulation (Yanagimachi and Chang, 1961). In the rabbit, which ovulates ten hours after coitus, only 51% of the eggs were fertilized when insemination took place at the time of ovulation (Chang, 1952a).

In addition to the short period of ovum viability leading to lowered fertility, increased embryonic or foetal death was found to be associated with the development of ova aged before fertilization. The first experimental evidence that late fertilization of ova was followed by retarded development of the resulting embryos was given by Blandau and Young (1939). In this study, the guinea pig was used. Fertilized aged ova showed cytolysis, blastomere fusion and retardation in the development rate. Embryos removed after uterine implantation showed gross structural abnormalities and were either undergoing resorption or were about to be aborted.

Other studies of the post-implantation period following delayed fertilization, resulted in reports of malformed embryos and fetuses with an increased incidence of abortion or resorption. Blandau and Jordan (1941) found an increase in the number of abnormal pregnancies in the rat, following fertilization of aged ova. They determined the upper limit of ovum age, from which any normal pregnancies resulted, to be about 10 hours after ovulation. The abnormal rat embryos and fetuses were resorbed before term. In a study of 24-day rabbit fetuses, following insemination eight hours later than normal, Chang (1952a) observed a foetal death rate of 59%. Also, there were six percent abnormal and only 35% normal fetuses. Iffy (1963) reported that in reviewing the literature on cases of pathological gestation in the human, in which the time of fertilization was known, 14 of 19 cases of abortion had been conceived after day 17 of the menstrual cycle.

The preimplantation stages of pregnancy following delayed fertilization have been mainly studied in the pronuclear stage of the ovum and the early cleavage divisions. However, Chang (1952a) examined 6-day blastocysts from rabbits which had been inseminated at the time of ovulation. Only 52% of 248 ova had developed into blastocysts and 12% of these were abnormal in size and cellular structure. In two-cell stages following delayed fertilization in the rat, (Blandau, 1952), fragmentation of nuclei was common. When hamsters were studied, it was found that 13% of aged ova failed to cleave at the two-cell stage and

22% at the four to eight cell stage. The failure rate was zero to four percent after normal fertilization (Chang and Fernandez-Cano, 1958). Hunter (1967) examined the early cleavage divisions in the pig and noted that a high proportion of eggs could be fertilized up to eight hours after ovulation, but failed at the four to eight cell stage.

Extra pronuclei have been reported to be present in a high proportion of eggs following delayed fertilization. The existence of more than two well-formed pronuclei in a fertilized ovum will lead to polyploidy in the resulting embryo. Three well-formed pronuclei were found in nine of 67 rabbit ova, when rabbits had been mated 10 hours after an ovulation-inducing injection of chorionic gonadotrophin (Austin and Braden, 1953). Also, hamster ova examined in the pronuclear stage when fertilization occurred five hours after ovulation, showed 34% trinuclear ova (Chang and Fernandez-Cano, 1958). Other species, in which an increased incidence of extra pronuclei were found after delayed fertilization of ova are the pig (Hancock, 1959; Thibault, 1959; Hunter, 1967), rat (Austin and Braden, 1954a; Odor and Blandau, 1956; Braden, 1958; Pikó, 1958) and the mouse (Braden and Austin, 1954; Marston and Chang, 1964).

Polyspermy, or the fusion of two or more male gametes with the female gamete, initiates the formation of two or more male pronuclei and has been associated with delayed fertilization. Austin and Braden (1953) in a classic paper on polyspermy, noted a sevenfold

increase in the incidence of polyspermy in normal outbred albino rats when mating was delayed. Odor and Blandau (1956) observed a rise of 11 times the normal 0.3% rate of polyspermy in delayed mating of Wistar albino rats. Braden (1958) undertook a study of strain differences on the effect of polyspermy in rats after delayed mating. He found that genetic variation influenced the results, although in every case, the proportion of polyspermic ova following delayed fertilization was substantially increased over that found after normal mating. This dependence on genetic background was confirmed by Piko' (1958). When various species were studied, it was found that the incidence of polyspermy following delayed mating was increased in some, but not in other species. In the pig, Hunter (1967) found an increase in polyspermy rising from 1.3% to 15.4% when ova were fertilized at four hours and 16 hours, respectively, after ovulation. However, no significant increase was noted in mice (Gates and Beatty, 1954; Braden and Austin, 1954; Braden, 1957; Marston and Chang, 1964), field voles (Austin, 1957) nor hamsters (Chang and Fernandez-Cano, 1958). The incidence of polyspermy after delayed mating did rise, however, when heat was applied locally to mice oviducts (Braden and Austin, 1954). Hyperthermia combined with delayed mating, was also found to increase further the number of polyspermic ova in the rat (Austin and Braden, 1954a; Austin, 1956; Piko' and Bomsel-Helmreich, 1960).

Polyspermy is usually prevented in mammals, mated under normal conditions, by either a reaction which the zona pellucida of the ovum undergoes after the entry of the first spermatozoon or by the vitelline block to polyspermy. The vitelline block is the name given to the change that occurs in the vitelline surface of the ovum after attachment of the first spermatozoon. After the block has developed, attachment to the vitelline membrane by other spermatozoa is prevented. The efficiency of the zona reaction varies with the species. For example, a highly efficient reaction is found in the golden hamster, field vole, dog, cow and sheep (Austin, 1960), so that only one spermatozoon usually penetrates the zona pellucida to the underlying perivitelline space. On the other hand, a hundred or more spermatozoa may penetrate into the perivitelline space in the rabbit (Braden, Austin and David, 1954; Dickmann, 1964; Austin and Walton, 1960). The block to polyspermy in this species is the vitelline membrane. Following a delay in mating and thus fertilization of aging ova, both zona and vitelline reactions to block the entry of a second spermatozoon deteriorate (Yanagimachi and Chang, 1961). Thus, supernumerary spermatozoa are able to penetrate the ovum and form extra pronuclei.

An extra pronucleus in the fertilized ovum may also be caused by the failure of emission of the first or second polar body with the result that the male pronucleus unites with extra female pronuclei. This abnormality is known as polygyny. The most common form of polygyny is

the suppression of the second polar body following fertilization. Unfortunately, it is often difficult to determine whether an extra pronucleus is derived from an extra spermatozoon or from the suppression of the second polar body. In some species, the male and female pronuclei are of different sizes, as in the rat (Austin and Braden, 1953), and therefore the origin of extra pronuclei may be determined. In other species, such as the rabbit, the pronuclei are of the same size (Austin, 1960). At times, an extruded second polar body may be seen together with three pronuclei, thus making a case for polyspermy. In a study of delayed fertilization in the golden hamster, Chang and Fernandez-Cano (1958) found an incidence of 35% of ova containing three pronuclei. In a later study, Yanagimachi and Chang (1961) observed cases of both polyspermy and polygyny in hamsters; four instances of each were recorded when insemination occurred six hours after ovulation. Thibault (1959) reported an incidence of 21% polygyny in pigs mated later than 36 hours after the onset of oestrus. However, Hunter (1967) found only one egg which could be attributed to polygyny in delayed mating of pigs; the remainder of the trinuclear ova were thought to be the result of polyspermy.


Regardless of whether extra pronuclei in a fertilized ovum are derived from the entry of more than one spermatozoon or from suppression of the second polar body, the resulting polyploid condition appears to be lethal. No known instance of homogeneous mammalian triploidy has

been found to survive the complete gestation period. Bomsel-Helmreich (1965) estimated death of triploid embryos to occur about mid-gestation.

III. THE EARLY EMBRYOLOGY OF THE RABBIT

The rabbit has been a favourite animal for use in embryological research since the days of de Graaf (1672). This has been due to the ease with which embryos at precise gestation times may be obtained, as the rabbit ovulates only after copulation. The following section deals with the significant facts leading to ovulation in the female and the early development of the fertilized ovum and embryo until the sixth day of gestation. The domestic rabbit has a gestation period of 32 days, with implantation of the blastocyst occurring on day seven.

Under domestic conditions, the female rabbit will mate with the male usually at any time of the year. No oestrous cycle in the true sense of the word occurs; however, a certain period of anoestrus due to environmental conditions has been noted. In England, does kept in unheated rooms at times experienced anoestrus during late autumn and winter (Hammond and Marshall, 1925), whereas the months from July to September, when temperatures were high, were reported to be poor breeding months in the United States (Asdell, 1964; Sittmann, Rollins, Sittmann and Casady, 1964).



In the wild state, on the other hand, a suggestion of a behavioural sexual cycle has been reported. Myers and Poole (1962), in a study of a confined wild population where females and males were running together at all times, observed a seven-day cycle at which time the females became sexually attractive to the males. This cycle was correlated with an associated one of follicular growth in the ovaries. Females, however, when held separate from the males entered into a state of persistent sexual receptivity. The authors concluded that the occurrence of a regular behavioural cycle appeared to be totally dependent on stimuli of a psychological nature occasioned by the presence of males.

Ovulation in the rabbit is not spontaneous but occurs approximately 10 hours after coitus (Barry, 1839; Heape, 1905; Hammond and Marshall, 1925; Walton and Hammond, 1928; Hill, Allen and Kramer, 1935; Austin and Braden, 1954b). The mating stimulus is transmitted neurohumorally to the anterior pituitary, which is activated to secrete gonadotrophic hormone. This stimulates growth of ovarian follicles and leads to rupture of the follicles and the shedding of ova. Reports have been published of female rabbits ovulating following mechanical stimulation of the vagina and/or cervix. However, Asdell (1964) reported this to be incorrect. In early work by Fee and Parkes (1929) and Smith and White (1931), in which the pituitaries were removed from female rabbits at timed intervals after mating, it was established that

ovulation would not occur if the pituitaries were removed within 90 minutes of mating. However, ova were shed at the usual time of 10 hours post-coitum if the operation was delayed beyond 90 minutes. Thus, it was established that sufficient gonadotrophin is released from the pituitary within 90 minutes of mating to cause maturation of the ovarian follicles and ovulation to occur at the usual time of 10 hours after mating.

The effect of exogenous gonadotrophins on the time of ovulation in the rabbit has been studied by a number of workers. Harper (1961; 1963) performed carefully timed experiments to determine the time of follicular rupture and expulsion of ova in relation to an injection of 25-50 I.U. luteinizing hormone (LH). It was found that ovulation occurred during the same period of time, whether it was induced by LH or normal mating. Fifty percent of the ovulations occurred between 10 1/2 to 10 3/4 hours and 100% by 14 hours post-coitum.

Oöcyte development and maturation has been the subject of numerous investigations. The following brief description gives the main features of this process. In the female rabbit, the gonad at birth still contains proliferating oogonia. It is not until the second to third week of post-natal life that the germ cells enter the first meiotic prophase (Teplitz and Ohno, 1963; Peters, Levy and Crone, 1965). Thus, the rabbit is different from most other Eutherian mammals, in which the female germ cells are already in first meiotic prophase at birth. By the

end of the third week of post-natal life in the rabbit, all oöcytes have completed up to the diplotene stage of meiosis and entered a resting period known as the dictyate. This is the stage of the primary follicle. In the mature, unmated female rabbit there is said to be a continuous production and regression of ripening follicles, in which the follicular cells divide and multiply (Hill and White, 1934).

Pincus and Enzmann (1935) gave a good description of the continued maturation of oöcytes within the ovary following copulation. At the time of mating, the average diameter of a ripening follicle is 970 μ and in some cases the oöcyte is in a mass of cumulus cells. Two hours later, tetrad formation is evident within the oöcyte nucleus and the liquor folliculi within the follicle has increased in amount. By four hours post-coitum, the nuclear membrane has disappeared and the first polar spindle with the tetrads located on the metaphase plate lies near the periphery of the oöplasm. The first polar body has formed by eight hours and rests between the zona pellucida and the vitelline membrane. The second metaphase spindle has formed below the surface of the membrane.

At ovulation, the secondary oöcyte is surrounded by four to eight layers of cumulus cells, known as the corona radiata (Dickmann, 1963). Usually, several eggs are massed together in a group of loosely suspended follicular cells, known as the cumulus mass (Chang, 1951a). By two hours after ovulation, in mated animals, the cumulus mass becomes more or less dispersed, although the corona cells are still closely

attached to the zona pellucida. By four hours after ovulation, the outer layers of corona cells have become torn off and spermatozoa may be seen in the perivitelline space (Chang, 1951a). In non-fertile matings, for example, when a vasectomized male is used, the ova may remain in the cumulus mass for seven or eight hours.

During the time the ova are maturing in the Graafian follicle prior to ovulation, the spermatozoa are undergoing maturation or capacitation within the female genital tract. Austin (1951) and Chang (1951b) reported that a minimum time of five or six hours was required before spermatozoa were capable of fertilizing rabbit ova. The term capacitation was put forth by Austin (1952) to denote the physiological change that spermatozoa undergo prior to penetration of ova in some species. Recently, Dziuk (1965) and Austin (1967a) suggested that an optimum capacitation time may exist which is closer to 10 or 12 hours. Capacitation is thought to enable the spermatozoon to undergo an acrosome reaction, which causes the release of hyaluronidase. This enzyme enables the spermatozoa to pass through the layers of cumulus cells which surround the ovum.

Once ovulation has occurred and the spermatozoa are capacitated, then fertilization will take place. Normally, fertilization of the ova is completed by 14 hours after copulation or four hours after ovulation (Pincus, 1931; Austin and Braden, 1954b; Chang, 1951a). The site of fertilization is in the ampulla-isthmus junction of the oviduct (Greenwald,

1961). The second meiotic division is completed after fertilization and the second polar body was noted to be present in all eggs by four hours after ovulation (Chang, 1951a). By six hours after ovulation, denudation of the corona radiata is complete and the male and female pronuclei are approaching one another within the ovum. Syngamy is reported to take place about eight hours (Chang, 1951a) and the first cleavage division between 11 and 14 hours after ovulation (Pincus, 1931; Gregory, 1930; Pincus and Enzmann, 1932; Chang, 1951a).

The oviduct of the rabbit secretes a layer of acid mucoprotein (Braden, 1952), known as the mucin coat, over the zona pellucida of the ovum. The time of deposition has been variously reported as anywhere from five or six hours after ovulation (Pincus, 1931; Hammond, 1934) to eight hours (Braden, 1952) or even 10-14 hours (Chang, 1951a). As the mucin coat is impenetrable to spermatozoa, its deposition has been said to be the main factor in limiting the time during which ova may be fertilized (Pincus, 1931; Hammond, 1934). However, the loss of the capacity of the ovum to be fertilized between six to nine hours after ovulation may also be due to the complete denudation of the cumulus cells surrounding the ovum (Chang, 1951a).

The development of the rabbit zygote from the first cleavage division to the blastocyst stage has been described in detail by Gregory (1930). The first cleavage division was found to occur around 22 hours 30 minutes post-coitum and the resulting two-cell stage could be found

in certain cases anywhere from 21 1/2 to 31 1/4 hours. No qualitative difference could be detected in the two cells, which were of equal size in most cases. The second cleavage division was estimated to take place about 25 1/2 hours post-coitum. The third cleavage division, resulting in the eight-cell stage, was found to take place around 32 hours 15 minutes after mating. By 40 hours 30 minutes, the eight cells had divided to form the 16-cell stage, now termed a morula. One of the 16 cells was found to be completely enclosed by the remaining cells. In addition, the mucin coat had increased in thickness. The blastomeres of the 16-cell stage were observed in mitosis about 47 hours post-coitum. Differentiation began with morphological changes becoming obvious between the outer cells, destined to become the trophoblast, and the inner cells, which form the inner cell mass. The inner cell mass eventually developed into the embryo proper. The morula persisted until a cleft appeared, which separated the cells of the trophoblast from those of the inner cell mass, about 75 hours post-coitum. The blastocyst stage had now been reached. The blastocyst entered the uterus about 80 hours post-coitum and enlarged by cell proliferation and expansion due to fluid accumulation in the blastocoele.

Daniel (1964a) studied the growth of trophoblast tissue and noted that the doubling time of the cells up to six days post-coitum was about eight hours, with the duration of mitosis approximately 30 minutes. By the sixth day after mating, the blastocyst had increased greatly in size and had an average diameter of 2.8 mm. and a volume of 11.5 mm.³.

Cross-sections of the six-day blastocyst showed the trophoblast to be one-cell thick, except where it was continuous with the inner cell mass. The inner cell mass was compact, about three cells deep in the thickest part, and lined the dorsal part of the cavity or blastocoele. About this time, the inner cell mass became flattened to form the embryonic disc. Implantation of the blastocyst was observed to occur around 168 hours post-coitum or on the seventh day after mating.

IV. MATERIAL AND METHODS

In order to study the effect of delayed fertilization on the number of chromosome abnormalities in blastocysts, a total of 36 adult female rabbits were successfully mated at timed intervals following an injection of chorionic gonadotrophin. The rabbits supplied to the laboratory were of diverse origin and previous reproductive history. The animals were caged individually for at least three weeks prior to use. An interval of 10 hours from the time of injection was taken as the time at which ovulation would occur. Also, as capacitation of rabbit spermatozoa has been reported to require a minimum of six hours, this was taken into account for the estimation of the time at which fertilization of the ovum was likely to take place.

Ovulation was induced by the injection of 25 I. U. chorionic gonadotrophin ("A.P.L." Ayerst Laboratories) into the marginal vein of the ear. Female rabbits were then mated to a normal male at intervals ranging from 0 to 14 hours after injection. At the time of mating, the female rabbit was taken to the male's cage. After mating had taken place, a post-coital vaginal smear was examined under the microscope for the presence of spermatozoa. Only when spermatozoa were seen was the mating recorded as positive. The doe was

returned to her cage immediately following mating. The number of animals used at the various intervals between injection of gonadotrophin and mating is given in Table 2.

Each female rabbit was killed by an overdose of Nembutal, six days after mating. The abdominal wall was cut and the uterine horns exposed. Clamps were placed at the junctions of the oviducts and uterine horns and also at the vaginal-uterine junction to prevent loss of blastocysts during manipulation and removal of the uterus. The ovaries were located and removed to enable counts of corpora lutea to be made. Next, the uterus, with clamps still in place, was taken out and placed into a dish containing saline warmed to 37°C.

Blastocysts were recovered by flushing each horn of the uterus with warm saline. An incision was first made near the utero-tubal junction below the first clamp. Next, a second cut was made at the vaginal-uterine end of the uterus. A 5 ml. syringe was filled with saline and the end inserted in the first incision, then the uterine horn was flushed out. This was repeated twice for each horn to ensure that all blastocysts would be removed. The blastocysts escaped through the opening made at the vaginal-uterine junction into a Petri dish.

After all the blastocysts had been collected, they were placed in a bottle containing 15 to 20 ml. of tissue culture medium 199 (Connaught Laboratories), to which Colcemid had been added to a

final concentration of 1/10,000. Colcemid will arrest mitosis at metaphase and thus enable chromosome counts to be made. The blastocysts were incubated in the above medium for 1 1/2 hours at 37°C. Following incubation, each blastocyst was placed in a separate centrifuge tube containing about 2 ml. hypotonic citrate (0.9% sodium citrate). Each tube was numbered and the blastocyst broken up by repeated aspiration with a Pasteur pipette. Separate pipettes were used for each blastocyst to prevent cells from one tube being transferred to another tube. Also, the pipettes and centrifuge tubes had been previously siliconized to lessen the number of cells adhering to the glass. Before the blastocysts were broken up, their size was noted as to average, small or very small size.

Following 10 min. incubation in the hypotonic citrate, the cells were centrifuged at 800 rpm for 5 min. The citrate was removed and the cells fixed in alcohol-acetic acid (3:1). The tubes containing the cells were refrigerated at 4°C overnight. The next day, the fixative was removed following centrifugation and 45% aqueous acetic acid added. A second change of acetic acid followed. The cells were resuspended in a few drops of acetic acid and dropped onto a slide warmed to 56°C on a hot plate. During the above procedure extreme care was taken to avoid undue loss of cells. The above method is a modification of the technique described by Ford and Woollam (1963).

The slides were stained with carbol fuchsin as recommended by Carr and Walker (1961). Preparations were examined under the oil immersion objective and thirty metaphase plates were counted, where possible, for each blastocyst to establish the chromosome number. Karyotypes were made from metaphase plates suitable for photography in which the chromosomes were well spread with few overlaps. High contrast copy film (Kodak) was used to photograph the metaphases. The film was developed in D-11 developer and enlargements of the chromosomes made on No. 5 Kodabromide paper. The chromosomes were cut out and arranged according to the classification of rabbit chromosomes given by Nichols et al (1965). In most cases, three karyotypes were made for each blastocyst, the morphology of the chromosomes was studied and the sex chromosome complex determined.

In addition to the 36 rabbits used in the experiment with delayed fertilization, 17 more rabbits were included in a study to determine the effect of various doses of chorionic gonadotrophin on the number of chromosome abnormalities. Eight of these rabbits received no hormone and five of them were injected with 0.25 ml. of 0.9% saline immediately prior to mating; the remaining three does were mated to the male without receiving any injection. The other nine rabbits were injected with the following doses of chorionic gonadotrophin immediately before mating: one rabbit received 50 I.U., two rabbits

200 I.U., two rabbits 300 I.U. and one rabbit 400 I.U. of gonadotrophin. Recovery and treatment of the 6-day blastocysts were identical to the method described previously.

V. RESULTS

A. The Number of Blastocysts Recovered Following Delayed Fertilization

A total of 251 blastocysts were recovered from the 36 female rabbits used in the study. When the number of 6-day blastocysts was compared to the number of ovulations which had occurred, as judged by a count of corpora lutea in the ovaries, a marked discrepancy was noted in the percent of blastocysts recovered at the longer time intervals between gonadotrophin injection and mating. The number of corpora lutea, however, was not influenced by the time interval. A mean ovulation rate of 10.4 ± 0.4 (range 5 to 16) was found. Figure 3 is a plot of the number of corpora lutea counted in the ovaries compared to the actual number of blastocysts recovered for each animal at the various delayed time intervals. Blastocysts were recovered from every animal mated at intervals from 0 to 10 hours after gonadotrophin injection. However, when the delay was greater than 10 hours, blastocysts were recovered from only four of the eight rabbits mated at these times. In all except one of these rabbits from which blastocysts were obtained, the number of blastocysts was considerably less than the number of ovulations. The one exception was

a rabbit mated 13 hours after gonadotrophin injection and which had nine corpora lutea in the ovaries and from which nine blastocysts were recovered. No blastocysts were found in the two remaining rabbits mated at the 13 hour delay interval.

The total number of corpora lutea and the total number of 6-day blastocysts recovered at the various intervals between gonadotrophin injection and mating are given in Table 2. One animal in the group mated immediately following injection had 12 blastocysts in the uterus on day six; however, a count of corpora lutea could not be made as one ovary was accidentally lost at autopsy. Therefore, the totals given in the table do not include either the number of corpora lutea or blastocysts from this animal. The percent of blastocysts recovered compared to the number of ovulations at the various time intervals is shown graphically in Figure 4. It may be seen that as the interval between injection and mating increased, the percentage of blastocysts found on day six decreased. Between 86% and 95% of ovulations were obtained as 6-day blastocysts when the interval between injection and mating was six hours or less. The percentage dropped to 76 and 63 percent of ovulations at eight and nine hour delay intervals, respectively. The greatest decline was found when mating took place at the time of ovulation or later, that is at intervals from 10 to 14 hours after injection. At 14 hours, only one blastocyst was recovered.

The blastocysts varied greatly in size, as judged by visual inspection before they were dissociated by aspiration with a Pasteur pipette. Although very small blastocysts, which were hardly visible to the naked eye, were found at all intervals, their frequency increased when the interval between injection and mating was four hours or more. Some of these very small blastocysts could not be studied cytogenetically as the nuclei appeared pycnotic and the mitotic rate was greatly diminished.

B. Chromosome Abnormalities Following Delayed Fertilization

Two hundred and twenty-seven or 90% of the 251 blastocysts recovered could be examined cytogenetically. Nineteen chromosome abnormalities were found. The cytogenetic feature of the chromosomally abnormal blastocysts are given in Table 3. For each anomaly, the number of cells counted, the distribution of chromosome number, the number of karyotypes prepared, the size of the blastocyst and a description of the abnormality are given. The following sections deal with the chromosome abnormalities found at the various delay-intervals between injection and mating. Rabbits mated immediately after injection of 25 I.U. gonadotrophin are included as a control group to the various delay-intervals.

(i) Blastocysts recovered from rabbits mated immediately after gonadotrophin injection

Seventy-three of the 75 blastocysts recovered from the nine

rabbits mated immediately following an intravenous injection of 25 I. U. chorionic gonadotrophin, were examined cytogenetically. Five chromosome abnormalities were found. The two blastocysts which could not be examined chromosomally were average in size. One was very granular in appearance on preliminary examination before it was dissociated in hypotonic citrate. The cells from this blastocyst were very small and pycnotic and few mitoses were observed. The other blastocyst, although of normal appearance when examined whole, was found to have very few cells with chromosomes in metaphase.

Two of the chromosomally abnormal blastocysts were mixoploid in nature. Both these blastocysts came from the same animal, were very small in size and had a proportion of the cells in the $8n$ or octaploid range, as well as a certain number of cells with the diploid number of chromosomes (Table 3, blastocysts 1a and 1b). In addition, cells containing the tetraploid number of 88 chromosomes were found in blastocyst 1a. Ten other blastocysts from this animal had a normal chromosome complement.

Another form of polyploidy was observed in a very large blastocyst from a second rabbit. In this instance, 18 of the 23 metaphases counted were in the pentaploid range of 110 chromosomes (Table 3, blastocyst 2a). A karyotype from this blastocyst is shown in Figure 5. The sex chromosome complex was XXXYY. The remaining five blastocysts from this animal had the normal diploid

number of 44 chromosomes.

The only instance of trisomy was found in this group of rabbits mated immediately after injection. One of four blastocysts from a rabbit was found to have a count of 45 chromosomes (Table 3, blastocyst 3a), the remaining three blastocysts were normal chromosomally. When the karyotype was assembled (Figure 6), an extra chromosome-1 was present. The sex chromosome complex was XY or male.

The fifth chromosome abnormality occurred in a blastocyst with a normal diploid count of 44 chromosomes, but with an abnormal karyotype (Figure 7). Six small acrocentric or chromosomes with a terminal centromere were present; a fragment seemed to be missing from the short arm of a medium-sized chromosome, perhaps chromosome number 3, and one of the medium-sized sub-terminal chromosomes was missing from pairs 16-18. This blastocyst was one of nine from rabbit four (Table 3, blastocyst 4a); the other blastocysts were normal in chromosome number and karyotype.

Thus, four of nine rabbits in this group had at least one chromosomally abnormal blastocyst with the abnormalities of a varied nature. The percentage of chromosome abnormalities in 6-day blastocysts recovered from rabbits mated immediately after gonadotrophin injection was in the order of seven percent.

(ii) Blastocysts recovered from rabbits mated two or four hours after gonadotrophin injection

Six day blastocysts recovered from rabbits mated either two or four hours after the injection of chorionic gonadotrophin did not differ greatly in either number or type of chromosome abnormalities. Therefore, these two delay-mated groups will be considered together. A total of 62 blastocysts (Table 2) were recovered; however, five of these were not suitable for chromosome study, four being of very small size and one blastocyst, although of average size, had small pycnotic cell nuclei and very few **mitoses**.

Two chromosome abnormalities were found in blastocysts taken from two hour delay-mated animals. The first anomaly was a $2n/4n$ mixoploid, the second a $43/44$ chromosome mosaic (Table 3, blastocysts 5a and 6a). The mixoploid blastocyst was very small in size, whereas the $43/44$ chromosome mosaic was within the normal size range. The karyotype shown in Figure 8 represents the chromosome complement of the normal diploid cells of the chromosomally mosaic blastocyst. The sex chromosome complex is female. Figure 9 is a karyotype from a cell with 43 chromosomes; one of the chromosomes is missing from pairs 7 to 9. If the missing chromosome is in fact number 8, or the X-chromosome, then the sex chromosome complex would be XO in the 43 chromosome cell population of this blastocyst. Thus the chromosome complement of the anomaly would be XO/XX.

A 43/44 chromosome mosaic blastocyst (Table 3, blastocyst 7a) was found also among the 6-day blastocysts recovered from rabbits mated four hours after gonadotrophin injection. In this instance, the normal diploid cell population, as shown in Figure 10, had a XY or male sex chromosome complex. In karyotypes (Figure 11) made from metaphases with 43 chromosomes, the missing chromosome was found to be one of the small acrocentric chromosomes. If the missing chromosome was the Y-chromosome, then the chromosome constitution of this blastocyst would be XO/XY.

The remaining 54 blastocysts recovered from animals mated two or four hours after gonadotrophin injection were normal in chromosome number and karyotype. The size of these blastocysts, except for three very small blastocysts, was within the normal range.

(iii) Blastocysts recovered from animals mated at six, eight or nine hours after gonadotrophin injection

The blastocysts obtained from animals mated at six, eight or nine hours after gonadotrophin injection are added together as they formed a uniform group as far as cytogenetic features were concerned. A total of 92 blastocysts were recovered at these intervals (Table 2). Of this number, 20 were observed to be smaller than normal in size. Eighty of the 92 blastocysts could be examined cytogenetically. The remaining 12 blastocysts had too few cells in mitosis to render an exact count of the chromosomes and karyotyping to be carried out.

The only chromosome abnormality found among the 80 blastocysts was triploidy. Ten blastocysts had the triploid complement of 66 chromosomes. The cytogenetic features for each triploid are given in Table 3. Seven of the triploid blastocysts were of XXY (Figure 12) and three of XXX (Figure 13) sex chromosome constitution. No triploids were found with an XYY sex chromosome complex. Two of the triploids were of small size, the remainder were within the normal size range. The incidence of triploidy in 6-day blastocysts recovered from rabbits mated from 6 to 9 hours after injection of chorionic gonadotrophin was thus in the order of 13%.

(iv) Blastocysts recovered from animals mated from 10 to 14 hours after injection of gonadotrophin

The rapid decline in number of blastocysts recovered when mating was delayed until the time of ovulation, at 10 hours, or later, resulted in only 22 blastocysts available for chromosome study (Table 2), from 10 animals used. Eight of the blastocysts were of small size and seven had a white opaque cast on visual inspection before dissociation. The number of cells in metaphase was reduced also. These blastocysts seemed to be in a state of abnormal development. However, 17 of the 22 blastocysts could be examined chromosomally. One chromosome abnormality was found. This occurred at the 13 hour delay-interval and was a 44/47 chromosome mosaic (Table 3, blastocyst 13a). The blastocyst was very small. Karyotypes made from metaphases containing the normal diploid complement

of 44 chromosomes (Figure 14) showed an XX or female sex chromosome complex. Three karyotypes with 47 chromosomes showed the extra three chromosomes to be similar to numbers 4, 6 and 19 (Figure 15).

C. The Sex Ratio of 6-Day Blastocysts Recovered from Rabbits following Delayed Fertilization

The sex chromosome complex was established for 226 blastocysts available for chromosome study. The number of blastocysts classed as having a male or female sex chromosome complex is given in Table 4. Blastocysts having an abnormal chromosome constitution were classed as having a male or female sex chromosome complex on the basis of the presence or absence of the Y-chromosome. For example, the pentaploid blastocyst with an XXXYY sex chromosome complex was placed in the column under the male heading.

Delay between injection of gonadotrophin and mating did not seem to influence the number of blastocysts with either a male or female sex chromosome complex. Thirty-nine of 72 or 54% of blastocysts recovered from rabbits mated immediately following gonadotrophin injection were found to have a male sex chromosome complex. This percentage did not change greatly with increased delay between injection and mating, except for the two hour delay interval. In this instance, one of the three rabbits produced 14 blastocysts, 12 of which had a male sex chromosome complex. As

a result the ratio for the number of blastocysts with a male sex chromosome complex at the 2 hour delay-interval is greater than at any of the other time intervals. When the total for all time intervals is taken, the percentage of blastocysts with a male sex chromosome complex is 54% and the percentage with a female sex chromosome complex is 46%.

D. Number of Hypodiploid, Diploid and Hyperdiploid Metaphases Following Delayed Fertilization

The number of metaphases that were hypodiploid, diploid and hyperdiploid in chromosome number were added for each time interval between injection and mating. Chromosome counts for the 19 chromosomally abnormal blastocysts were excluded. A total of 4,076 metaphase plates are represented in Table 5.

The percentage of metaphases in which the number of chromosomes was less than the normal diploid number of 44 ranged between 32% and 43%. The missing chromosomes were probably lost when some of the cell membranes ruptured during the preparation of the blastocysts for chromosome study. No significant difference was found when the various time intervals were tested ($p > 0.05$). The number of diploid metaphases in which the exact number of 44 chromosomes was found ranged between a lower limit of 53% and an upper limit of 64%. Very few metaphases with a greater number of chromosomes than 44 were found.

E. Effect of Various Doses of Chorionic Gonadotrophin Injected Immediately Prior to Mating on the Number of Chromosomally Abnormal Blastocysts

The intravenous injection of chorionic gonadotrophin in doses of 25 to 300 I. U. immediately before mating resulted in ovulation occurring in each of the 16 rabbits used. In addition, two rabbits, one receiving 200 I. U. and the other 400 I. U. HCG, were found to be intersexes at autopsy and therefore are excluded from this group. These two animals have been described elsewhere (Shaver, 1967). Only six of the eight rabbits receiving no exogenous gonadotrophin ovulated, although each animal mated and the presence of motile spermatozoa in the vagina was verified.

The percentage of ovulations recovered as 6-day blastocysts was found to be influenced by the dose of chorionic gonadotrophin (Table 6). A normal rate of recovery of 86% to 100% was obtained when the dose of HCG injected was between 25 to 100 I. U. However, with the higher doses of 200 and 300 I. U. HCG, the rate of recovery dropped to 64% and 35%, respectively.

The control group of six rabbits, which ovulated but were not injected with chorionic gonadotrophin, yielded 54 blastocysts. Fifty-two of these could be examined chromosomally. One chromosome abnormality was found, giving an incidence of two percent chromosome abnormalities for this group. The chromosomally abnormal blastocyst was a 44/45 chromosome mosaic (Table 7, blastocyst 1a).

Figure 16 is a karyotype from the cell population with the normal diploid number of 44 chromosomes. The sex chromosome complex was XY. An additional small acrocentric chromosome was present in the karyotypes from metaphases with 45 chromosomes (Figure 17). It was impossible to determine whether the extra acrocentric chromosome was similar to autosomes 21, 22 or the Y-chromosome. In the karyotype (Figure 17), the extra acrocentric chromosome was placed arbitrarily in autosome position 22.

Chromosome abnormalities found in blastocysts recovered from the nine animals mated immediately following an injection of 25 I. U. chorionic gonadotrophin have been described in a previous section. The cytogenetic features of the five chromosomally abnormal blastocysts found in this group are listed again in Table 7. The representative karyotypes are to be found in Figures 5-7 for the pentaploid, trisomy-1 and the diploid with 6 small acrocentric chromosomes. The incidence of 7% chromosomally abnormal blastocysts in this group is not significantly different from the 2% incidence found in the rabbits not receiving an ovulating dose of gonadotrophin ($p > 0.05$).

Two additional chromosome abnormalities arose among 28 blastocysts recovered from animals which had received 50 and 75 I. U. HCG immediately before mating. Both of the chromosomally abnormal blastocysts were 43/44 chromosome mosaics (Table 7,

blastocysts 6a and 7a). Karyotypes prepared from metaphases with 44 chromosomes revealed an XY sex chromosome complex in both blastocysts (Figures 18, 20). Cells which contained 43 chromosomes had one of the small acrocentric chromosomes missing in each of the two chromosomally abnormal blastocysts (Figures 19, 21). Again, it was impossible to determine whether one of the small acrocentric autosomes or the Y-chromosome was the missing chromosome. If the Y-chromosome was absent in the cells with 43 chromosomes, then the sex chromosome complex would be XO/XY for the chromosome mosaics. If it was autosome 21 or 22 that was missing, then monosomy-21 or monosomy-22 would be the chromosome constitution of the cells with 43 chromosomes.

No chromosome abnormalities were found among 24 blastocysts recovered from rabbits receiving doses of 100-300 I.U. HCG (Table 6).

VI. DISCUSSION

A. The Effect of Delayed Fertilization on the Number of 6-Day Blastocysts Recovered

The decline in the number of 6-day blastocysts recovered from rabbits, in which mating had been delayed at least 6 hours, may be attributed to a combination of factors. The major cause of the decline in number is the short time after ovulation during which rabbit ova may be fertilized. Furthermore, a certain number of fertilized ova may be lost through failure of segmentation or blastocyst formation to proceed normally.

Although chorionic gonadotrophin was used to induce ovulation in this study of delayed fertilization in the rabbit, the mean number of 10.4 ovulations is in excellent agreement with other investigations, in which ovulation was induced by a normal mating alone or in conjunction with an injection of gonadotrophin. For large breed rabbits, mean ovulation rates have been reported as follows: 10.7 (Venge, 1950), 10.5 (Adams, 1958), 10.3 (Adams and Chang, 1962). Harper (1963) using an injection of 25 I.U. luteinizing hormone to induce ovulation, reported a mean number of 11.0 ovulations. In this instance, no mating or insemination was carried out. The number of ovulations at any one

time is known to depend on the size of the breed of rabbit. Gregory (1932) observed a mean ovulation rate varying from 3.97 for the small Polish breed to 12.88 for the large breed Flemish Giant. The rabbits used in the present experiments, although of mixed breed, were of large size and varied in weight from 2.7 - 4.6 Kg.

The combination of the ovulation-inducing dose of 25 I. U. chorionic gonadotrophin injected into the rabbit, plus any endogenous pituitary gonadotrophin that might be released at the time of mating, did not seem to influence the average number of corpora lutea found. No correlation was found when the number of corpora lutea was tested against the delay between injection of gonadotrophin and mating (correlation coefficient of 0.0). The only influence the chorionic gonadotrophin appeared to have was that the minimum level of circulating gonadotrophin was present for ovulation to occur, as it did in every rabbit injected. This was not the case in two of the rabbits injected with saline and mated to a normal male. In these two instances, apparently the blood level of pituitary gonadotrophin following mating was not sufficient to induce ovulation, as no corpora lutea were found in the ovaries of these rabbits.

The number of ova actually fertilized, however, depends to a great extent on the time at which mating or insemination takes place in relation to the time of ovulation. The rabbit ovum seems to have an upper limit of approximately 10 hours after ovulation for the chance of fertilization to occur. Only one 6-day blastocyst was recovered when

mating took place 14 hours after gonadotrophin injection or four hours after the assumed time of ovulation. If a minimum time of six hours is required for capacitation of spermatozoa, then fertilization, in this case, would occur at 20 hours post-coitum or 10 hours post-ovulation. Hammond (1934), in a study of litter size at birth following delayed fertilization, concluded that the upper limit was six hours after ovulation. Chang (1952b) found that rabbit ova were capable of being fertilized up to eight hours after ovulation, although the number of ova fertilized dropped sharply after four hours. Braden (1952), also arrived at eight hours as the time after ovulation during which rabbit ova could be fertilized. Adams and Chang (1962) studied ova from rabbits mated from eight to 14 hours after an injection of luteinizing hormone. The animals were sacrificed between 25 and 40 hours later and the ova examined before and after staining for signs of fertilization. An upper limit of 13.5 hours after mating was determined as the time at which ova lost the capability to be fertilized. This time limit is in very close agreement to the 14 hours, approximately, found in the present study.

The eight to 10 hour post-ovulation life of the rabbit ovum, during which fertilization can occur, is short when compared to other laboratory animals which have been studied experimentally. The life of the guinea pig ovum has been estimated to be about 20 hours (Blandau and Young, 1939), the rat ovum, about 12 hours (Blandau and Jordan, 1941; Blandau, 1952), the mouse ovum, approximately 15 hours, (Marston and Chang, 1964) and that of the golden hamster, about 12

hours (Yanagimachi and Chang, 1961). The ferret, an animal in which coitus induces ovulation, has a relatively long period of 30-36 hours during which the ovum may be fertilized (Hammond and Walton, 1934; Chang and Yanagimachi, 1963). There is a great species variation, therefore, in the length of time the ovum remains viable.

The above times, however, are the upper limits of ovum life and a drastic decline in number of fertilized ova usually occurs prior to the limit. It was found that a rapid decline in the number of 6-day blastocysts occurred when mating took place at the time of ovulation, that is 10 hours after gonadotrophin injection. In this instance, ova would have been aged approximately six hours, allowing time for capacitation, before fertilization. A discrepancy is noted when these results are compared with those obtained by Chang (1952a) and Adams and Chang (1962). These investigators found that 51%-57% of rabbit ova were fertilized when insemination took place at the time of ovulation. Individual variation, both within animals and between animals, is known to occur in the ovulation time of rabbits (Harper, 1961, 1963). This is reflected in the present work in the four rabbits mated 11-14 hours post-injection, in which no blastocysts were recovered, and the rabbit mated at 13 hours, in which nine blastocysts were recovered from nine ovulations. Also, a certain number of fertilized ova may not survive to the 6-day blastocyst stage.

An increase in the number of small blastocysts, which did not seem to be developing at the normal rate, was noted as the delay

between gonadotrophin injection and mating increased. Loss of blastocysts due to failure of the early cleavage divisions or blastocyst formation could also account for a portion of the decline in number of blastocysts recovered on day six.

Considerable prenatal mortality occurs in animals under normal conditions. Approximately 30% of zygotes are lost before birth in the domestic rabbit (Adams, 1960) and 43% in the wild rabbit (Brambell, 1948). Hanly (1961), in a review of prenatal mortality in pigs, estimated that 33% of the embryos died during the first half of gestation. Braden (1959) reported the proportion of ovulated ova that were lost before the end of gestation in rats to be 16.7%. In the human, 15% of foetuses were estimated to be lost as spontaneous abortions (Roth, 1963; Warburton and Fraser, 1964).

Few studies have been made of the loss before implantation as most investigations include the implantation period. They are based on a comparison of the number of corpora lutea and the number of implantation sites in the uterus. This would include failure occurring at fertilization, cleavage, blastocyst formation and growth, as well as implantation. In the domestic rabbit, Adams (1960) estimated a pre-implantation loss of 11.4% and the loss in the wild rabbit was given as 10.2 - 13.0% by Brambell (1948).

Delayed mating considerably increases the amount of zygotic loss. Chang (1952a) studied 24-day rabbit foetuses from eight hour delayed-matings and determined the preimplantation loss to be in the

order of 60%. Braden (1959) found a loss of 49.2% in late-mated rats. Twenty-seven percent of this loss was attributed to non-fertilization of ova; 14.6% to loss between fertilization and implantation; and 7.5% through cases of abnormal fertilization. A further loss of 18.8% occurred after implantation.

The loss before implantation in the present study varied from 5% at delay-intervals of two hours to 90% at 14 hours delay. This would include ova not fertilized and failure at cleavage or blastocyst formation and growth. The 14% loss found at the zero-delay interval may be influenced to some extent by excess gonadotrophin in the peripheral blood of the rabbits mated immediately after injection and will be discussed later.

B. Chromosome Abnormalities Found in 6-Day Blastocysts Following Delayed Fertilization

The chromosome abnormalities observed in 155 blastocysts from the 27 rabbits, in which mating had been delayed from two to 14 hours after gonadotrophin injection, consisted of 11 polyploids and three chromosome mosaics. In addition, 73 blastocysts from nine rabbits mated immediately following injection yielded three polyploids, one trisomy and one blastocyst with 44 chromosomes but having an abnormal karyotype. The actual number of chromosome abnormalities was not significantly different statistically when the various groups were tested. That is, delay in fertilization did not produce a significantly greater number of chromosome abnormalities. However, when

the type of chromosome abnormality was tested, the incidence of triploidy was significantly greater at delay-intervals of six to nine hours than when there was no delay between gonadotrophin injection and mating ($p < 0.001$).

Rabbit ova with three pronuclei, presumably triploid, have been observed by other investigators following delayed fertilization. Austin and Braden (1953) found 16.4% trinuclear ova when insemination took place 10 hours after an ovulation-inducing injection of gonadotrophin, compared with 1.4% trinuclear ova in control rabbits mated at the normal time. Although Austin and Braden (1953) attributed the extra pronucleus in the ovum to polyspermy, no supernumerary sperm tails or mid-pieces were seen within the ovum and the possibility that polygyny contributed to the trinuclear ova cannot be overlooked. In repeating the experiment, Austin (1960) found trinuclear ova present only in rabbits mated one and two hours after ovulation had occurred. Not one ovum with an extra pronucleus was found when mating took place before the time of ovulation. Adams and Chang (1962) examined ova for polyspermy and found only one doubtful case among 28 pronuclear eggs from rabbits mated at either 11 or 13 1/2 hours after LH injection. They concluded that polyspermy was not increased after delayed mating in the rabbit.

The 10 triploid blastocysts recovered in the present series were taken from rabbits mated six to nine hours after gonadotrophin injection. Ova, therefore, would be aged two to five hours, approxi-

mately, before fertilization occurred. When Figure 4 is examined, the six hour interval between injection and mating is found at the start of the decline in the number of blastocysts recovered, and may be the time at which ova, when fertilized, show the earliest signs of ageing. When the sex chromosome complex of the triploid blastocysts was examined, seven blastocysts were XXY and three blastocysts XXX. No XYY triploids were found. Therefore, polyspermy following delayed fertilization in the rabbit does not seem to be the major factor in the production of triploidy. Suppression of the second meiotic division probably is the more frequent cause of triploidy in rabbit blastocysts following delayed mating.

Triploidy, determined by actual chromosome counts in developing embryos, has been described following the application of heat to the oviduct of the female mouse at the time of mating. Heating of ova was accomplished by pulling the oviduct outside the body and surrounding it with circulating water heated to 45°C. When the 3 1/2 day blastocysts were examined, approximately 11% of the embryos were of a triploid chromosome constitution (Beatty and Fischberg, 1949; Fischberg and Beatty, 1952). Second polar body suppression was thought to be the cause. In the rat, heat applied to the oviduct at the time of mating gave only temporary suppression of the second polar body; the main effect was to induce polyspermy (Austin and Braden, 1954b). A species difference, therefore, seems to exist with the application of heat as a means of second polar body suppression. It

has been suggested that the application of heat might slow down the transport of ova in the oviduct and result in delayed fertilization in these cases (Beatty, 1957).

Spontaneous triploidy has been found in certain strains of mice (Beatty and Fischberg, 1949, 1951; Beatty, 1957). Matings within certain strains resulted in a low incidence of triploid embryos but matings between strains gave a significantly greater number of triploid blastocysts. Braden (1957) suggested that the loss of the capacity of the ovum to produce a second polar body was under genetic control to some extent.

Colchicine treatment at the time of fertilization has been another experimental means to induce triploidy in embryos by interference with the second meiotic division of the ovum. Edwards (1954, 1958a) injected colchicine into the mouse uterus just prior to mating, then examined the embryos at 3 1/2 days. Large numbers of heteroploids were found, of which half were triploid. However, in addition to the heteroploid embryos, androgenic haploids arose due to all the female chromatin being expelled into a polar body, leaving the male pronucleus in the ovum. The androgenic haploids, however, were lost at the first cleavage division. Pikó and Bomsel-Helmreich (1960) observed that the time of colchicine injection was important. When injection was done, two hours after mating, suppression of the second polar body occurred; at 2 1/2 hours, all the female chromatin was expelled into the polar body. The chemical diacetylmethylcolchicine

(Colcemid) was found by Edwards (1961) to be more effective and less toxic than colchicine, and resulted in more triploids and fewer cases of complete loss of maternal chromatin. Colcemid and in vitro fertilization were combined to cause a 97% incidence of trinuclear ova, due to suppression of the second polar body, in the rabbit by Bomsel-Helmreich (1965). The fertilized ova were then transferred to recipient does for further development in order to study the survival of triploid embryos during gestation.

Triploid embryos and fetuses are known to survive implantation. Fischberg and Beatty (1951) found triploid embryos at 9 1/2 days gestation in the mouse, which is approximately mid-term in the gestation period. The triploids appeared normal in development, although slightly smaller than diploid litter-mates. In the rat, triploid embryos were observed at 12 days gestation and all of them seemed to be retarded in growth. At 13 - 15 days, no triploids were found, although a few diploid/triploid chromosome mosaics were observed (Piko' and Bomsel-Helmreich, 1960). In the study of triploid rabbit embryos by Bomsel-Helmreich (1965), the mean diameter of triploid blastocysts was smaller than diploid blastocysts on the seventh day of gestation. The triploids were found to implant a few hours later than the diploid embryos. Following implantation, some triploids went to mid-gestation with mortality growing progressively greater as the time between implantation and the 15th day increased.

In a study of chromosome abnormalities in early human spontaneous abortions, Carr (1963, 1965) found nine cases of triploidy in 200 abortions for an incidence of 4.5%. Other instances of triploids in human abortions have been reported (Delhanty, Ellis and Rowley, 1961; Szulman, 1965; Thiede and Salm, 1964; Penrose and Delhanty, 1961; Schlegel, Neu, Leão, Farías, Aspillaga and Gardner, 1966; Waxman, Arakaki and Smith, 1967; Inhorn, Therman and Patau, 1964; Edwards, Yuncken, Rushton, Richards and Mittwoch, 1967). Homogeneous triploidy therefore appears to be lethal in humans also, at or before mid-gestation. The sex chromosome complex from a total of 22 triploids from these reports gave 13 XXY, 6 XXX and 3 XYY. It is interesting to compare this ratio with that found in the rabbit. In both species, there were twice as many XXY as XXX triploids. The XYY sex chromosome complex is much less frequent, being about one half the number of XXX triploids in the human. As in the rabbit, the distribution of the sex chromosome complement varies from what would be expected if two male gametes (polyspermy) united with the female pronucleus. The ratio, in this instance, would be 1:2:1 of XXX:XXY:XYY. Neither can it be explained on the basis of suppression of the second polar body alone, as this would give a 1:1 ratio of XXX:XXY. Triploidy may also arise from aneugamy. In this case, the female pronucleus would be diploid in chromosome number due to suppression of the second meiotic division before anaphase separation of the chromosomes. Then the ovum, fertilized by a normal haploid spermatozoon would be triploid. This would

lead, also, to a 1:1 ratio of XXX:XXY sex chromosomes.

Although homogeneous triploidy appears to be lethal around mid-gestation, cases of diploid/triploid mosaicism are known to survive the latter part of gestation and birth in the human. Five individuals have been described with a diploid cell line present in blood or bone marrow (Böök, Masterson and Santesson, 1962; Ellis, Marshall, Normand and Penrose, 1963; Ferrier, Ferrier, Stolder, Bühler, Bamatter and Klein, 1964; Johnston and Penrose, 1966; Schmid and Vischer, 1967). Böök et al (1962) suggested that the haemopoietic system had to be diploid to support life. Bomsel-Helmreich (1965) noted that triploid mortality was greatest at the time yolk-sac circulation was being replaced by allantoic circulation. Recently, however, a report was published of a 7 1/2 month human infant that survived a few hours following birth, who showed XXY triploidy in all metaphases studied from a leucocyte culture (Bernard, Stahl, Coignet, Giraud, Hartung, Brusquet and Passeron, 1967).

The most interesting example of polyploidy found during the study of the effect of delayed mating on chromosome abnormalities was the pentaploid blastocyst. In this case, spermatozoa should have been fully capacitated at the time of ovulation, as the animal was mated immediately following injection of gonadotrophin. Pentaploidy, by actual chromosome count, has not been described previously in a mammal, although it has been reported in Amphibia (Beatty, 1957). The route Beatty (1957) suggested as the most likely to result in pentaploidy

involved suppression of the first and second meiotic divisions of the ovum plus fertilization by a haploid spermatozoon. The sex chromosome constitution would then be XXXXX or XXXXY depending on whether the tetraploid ovum was fertilized by an X- or Y-bearing spermatozoon. The sex chromosome complex found in the pentaploid blastocyst was XXXYY, therefore, it could not have arisen from suppression of the first and second meiotic divisions. Various possibilities exist to explain the sex chromosome complex of the pentaploid. Polyspermy may have resulted in two X-bearing and two Y-bearing spermatozoa fertilizing a haploid ovum. Also, polyspermy combined with polygyny could be involved. In this case a diploid ovum would be fertilized by one X- and two Y-bearing spermatozoa. Another possibility would be the inhibition of the first mitotic division of a normal diploid XY zygote to give the tetraploid XXYY constitution and the incorporation of the second polar body or another sperm into the zygote. Fourthly, cytoplasmic fusion of a triploid XXY ovum with a normal XY ovum might be the cause of the pentaploid condition. Fusion of two separately fertilized ova has been reported to occur (Tarkowski, 1961; Mintz, 1962; Malouf, Benirschke and Hoefnagel, 1967).

Three mixoploid blastocysts were found at the shorter time intervals between injection and mating. The early cleavage divisions must have been suppressed to give such combinations as $2n/4n$ and $2n/4n/8n$. Mixoploids of the $2n/4n$ variety have been reported after the application of colchicine (Edwards, 1958b) and heat (Beatty, 1967) during

the early cleavage stages of the mouse. Tetraploidy was found in approximately one percent of the metaphases from blastocysts with a predominantly diploid number of chromosomes. In the mixoploid blastocysts at least 30% of the metaphases were tetraploid or octaploid in chromosome number.

Chromosome mosaics, in which the diploid cell population was found to be present with a hypo- or hyperdiploid cell line were observed at the two, four and 13 hour delay-intervals. Austin (1967b) examined the chromosomes of the metaphase plate of the second maturation division in unfertilized rabbit ova at three and seven hours after ovulation. In 34 ova recovered from rabbits at three hours, the chromosomes were all normal in arrangement. At seven hours after ovulation, the chromosomes had scattered and two or three could be seen detached from the remaining chromosomes in 18/67 ova. Only 29/67 eggs had the normal chromosome arrangement. When 6-day blastocysts were examined from rabbits inseminated at the normal time, Austin (1967b) found that the majority of chromosome counts were diploid, with only 26% of the metaphases classed as hypodiploid, having from 38 to 43 chromosomes. In rabbits inseminated eight hours after an injection of chorionic gonadotrophin, 63% of the chromosome counts were hypodiploid. It was suggested that the chromosome derangements seen in ova at seven hours post-ovulation were due to ageing effects. The counts of hypodiploid chromosome numbers in the 6-day blastocysts recovered from rabbits mated eight hours after gonadotrophin injection, in the

present study, yielded only 35% of the metaphases with fewer than 44 chromosomes (Table 5). This was not significantly different from the control rate of 32%. At the 10-14 hour delay-interval, 42% of the metaphases were hypodiploid, a slightly greater proportion. The 44/47 chromosome mosaic blastocyst found at the 13 hour delay-interval might have arisen from non-disjunction or anaphase lagging at the second meiotic division of the ovum or the early cleavage divisions of the zygote. The chromosome abnormalities found at the two and four hour delay-intervals were both 43/44 chromosome mosaics. These would arise from ova fertilized shortly after ovulation and in which severe ageing changes would not likely be present.

It was difficult to determine whether a sex chromosome or an autosome was missing in the 43 chromosome population of the mosaic blastocysts. The X-chromosome is similar in morphology to chromosome pairs 9 and 10 and the Y-chromosome to pairs 21 and 22. In the studies of chromosomes in spontaneous abortions, 21% of 153 chromosomally abnormal abortuses were found to have a chromosome missing from group C, the group that contains the X-chromosome in man (Standardization of procedures for chromosome studies in abortion, 1966). As most of these abortuses were chromatin-negative when sex chromatin was analysed, the karyotype was thought to be XO. The XO condition of the human is known as Turner's syndrome and is associated with sexual infantilism in the adult female. A high rate of prenatal mortality is associated with the XO fetus in the human (Carr, 1965). In

contrast to man, the XO condition in the mouse is usually viable and fertile (Russell, Russell and Gower, 1959). A spontaneous occurrence of 0.1 to 1.7% XO mice was found according to the genetic strain (Russell and Saylor, 1961). The incidence was increased by 100r irradiation after sperm penetration of the vitelline membrane (Russell and Saylor, 1960). However, no evidence of XO mosaicism was found with irradiation and it was suggested that the loss of the sex chromosome occurred before the first cleavage division.

The two remaining chromosome abnormalities in the experiments with delayed mating were found in blastocysts from rabbits mated immediately after hormone injection. A trisomy for the largest chromosome of the rabbit karyotype was the only example of trisomy found during the study. Autosomal trisomy is frequently found in cases of spontaneous abortion and seems to be associated with advancing maternal age (Lenz, Pfeiffer and Tünte, 1967). Few cases of autosomal trisomy have been reported in living experimental animals. Cattanaach (1964) and Griffen and Bunker (1964) observed autosomal trisomy for one of the smallest chromosomes in the mouse. Butcher and Fugo (1967) found two autosomal trisomies in a group of 11-day rat embryos derived from ova delayed in the time of ovulation.

The unusual karyotype of the blastocyst having a count of 44 chromosomes but lacking a chromosome from numbers 16-18, an arm from chromosome 3 and having an extra small chromosome, may have arisen from a break in chromosome 3 or a deletion in chromosomes 16-18

forming an extra fragment and a loss of the remainder of the middle-sized chromosome through non-disjunction or anaphase lagging at the first cleavage division.

The variety in the type of chromosome anomalies found in the group of rabbits mated immediately following gonadotrophin injection led to the experiments utilizing various doses of chorionic gonadotrophin from 0 to 300 I.U., injected into rabbits immediately prior to mating.

C. Effect of Human Chorionic Gonadotrophin on 6-Day Rabbit Blastocysts

Coital stimulus in the rabbit causes the release of pituitary gonadotrophin which in turn induces ovulation. Ovulation may also be produced experimentally by the injection of LH or HCG. The minimal dose of the exogenous hormones needed to cause ovulation is 25-30 I.U. However, the amount of endogenous hormone released by the pituitary at mating is not known. The rabbit ovary responds to circulating gonadotrophin by releasing progesterone (Forbes, 1953; Hilliard, Hayward and Sawyer, 1964). The progesterone, in a feed-back mechanism, inhibits further release of gonadotrophin. Forbes (1953) assayed the pre-ovulatory progesterone in the peripheral blood of rabbits receiving an intravenous injection of gonadotrophin and compared the progesterone blood level to that found in rabbits following mating. The first detectable amount of progesterone was found at 64 min. after administration of gonadotrophin or 100 min. after mating. It was noted that after the progesterone appeared in the peripheral blood, its concentration fluctuated

widely and with an irregular pattern during the first day and a half following administration of gonadotrophin or mating. Hilliard et al (1964) perfused the ovarian artery of a rabbit with blood from mated donors and observed an increased level of progesterone in the venous effluent. The circulating gonadotrophin was found to remain elevated for approximately six hours post-coitum. The progesterone blood level remained elevated until two to three hours prior to ovulation.

The injection of chorionic gonadotrophin followed immediately by mating could possibly result in pituitary gonadotrophin being released as well, because the progesterone blood level would not be sufficient at the time of mating to inhibit release of pituitary gonadotrophin. Thus, in the present study, increased levels of gonadotrophin and progesterone may be present in the peripheral blood of rabbits mated immediately after gonadotrophin injection.

The study of 6-day blastocysts recovered from rabbits mated immediately following doses of 0 - 300 I.U. chorionic gonadotrophin revealed that the number of blastocysts recovered declined with doses of 200 and 300 I.U. Furthermore, chromosome abnormalities were found in 7% of the blastocysts from animals injected with 25-75 I.U. HCG prior to mating, whereas, only 2% of the blastocysts from rabbits receiving no exogenous gonadotrophin had chromosome abnormalities. Increased doses of chorionic gonadotrophin did not increase the number of chromosome abnormalities.

Recently, Soupart (1966) studied the effect of human chorionic gonadotrophin on capacitation of rabbit spermatozoa. Initially, the spermatozoa were capacitated in rabbits, which had received doses varying from 25 to 400 I.U. HCG, then, 10 hours later a bilateral hysterectomy was performed. The spermatozoa, capacitated under the various doses of gonadotrophin, were transferred to the oviducts of does which had received an ovulation-inducing injection of 25 I.U. HCG 12 to 13 hours previously. Ova were recovered from these does 13 to 14 hours later and examined for signs of fertilization. The percentage of fertilized ova was drastically reduced when spermatozoa had been capacitated in rabbits receiving 100 - 400 I.U. HCG. At doses of 400 I.U., total inhibition of capacitation was found. The decrease in the number of six-day blastocysts recovered from rabbits injected with 200 and 300 I.U. HCG at the time of mating, therefore, was probably due to a proportion of ova not being fertilized through failure of spermatozoa being fully capacitated.

Excess gonadotrophin and steroid hormones have been reported to induce changes in developing ova. Anomalies of the zona pellucida of the baboon were found in animals treated with superovulation amounts of gonadotrophin, as compared with untreated animals (Katzberg and Hendrickx, 1966). Vesiculation, hypotrophy or absence of the zona pellucida was noted in the treated animals. However, a study by Maruffo (1967) using Rhesus monkeys, only showed changes in the zona pellucida when examination was done with the electron microscope. No change in

the zona pellucida could be detected with the light microscope.

Studies on the effect of various steroids on rabbit ova have been reported. Daniel (1964b) found that progesterone inhibited cleavage of rabbit ova in vitro and that estrogen either inhibited cleavage or caused ova to fragment. Daniel and Cowan (1966) reported several of the steroids in oral contraceptives interfered with normal cleavage of rabbit ova in vitro. Three effects were seen, complete inhibition of cleavage, retardation of cleavage and fragmentation or the division of the egg into small spherical bodies. However, Daniel and Cowan (1966) stressed the fact that the dose levels used in vitro were much greater than what would be expected in vivo. Chang and Yanagimachi (1965) and Greenwald (1967) observed that estrogen injected into rabbits shortly after mating caused acceleration of ova through the oviduct or caused retention of ova for longer than normal at the ampullary-isthmic junction of the oviduct, depending on the dose of estrogen used. Lower doses of estrogen caused acceleration, higher doses, tube-locking. Increasing progesterone levels result in retardation of egg transport through the ampulla due to a depressing effect on muscular activity (Blandau, 1967).

An increased incidence in chromosome abnormalities has recently been reported in abortions from women, who became pregnant after taking oral contraceptives (Carr, 1967). Four triploid or diploid/triploid and two XO abortuses were found in eight abortuses examined chromosomally. Thus, hormone imbalance may be an important cause of chromosome abnormalities resulting in foetal loss.

D. Sex Ratio

The primary sex ratio, that is the number of ova fertilized by X- and Y-bearing spermatozoa is not known for any mammal. Various studies have been made to determine the ratio of males:females at birth and during gestation. The greatest amount of information is based on surveys in the human. The ratio at birth, for the human, is approximately 106:100 males to females (Stern, 1960).

Recent studies on the chromosomes of spontaneous and induced abortions have resulted in a sex ratio of 88 XY per 100 XX diploid abortuses for the surveys of spontaneous abortion, and 92 XY per 100 XX diploid abortuses from induced abortions (Standardization of procedures for chromosome studies in abortion, 1966). These figures did not include the chromosome abnormalities, which should be incorporated in the estimate of the primary sex ratio. From these studies, the primary sex ratio appears to be near equality.

In contrast to the above, surveys on spontaneous and induced abortions, using the sex chromatin technique to determine the sex, gave ratios considerably in excess of 1:1, with many more males than females found (Tricomi, Serr and Solish, 1960; Serr and Ismajovich, 1963; Szontágh, Jakobovits and Méhész, 1961). However, chromosome abnormalities such as XO would be classed as chromatin-negative and a proportion of the XXY triploids would be counted as chromatin-positive.

One study of the preimplantation period was made in golden hamsters (Sundell, 1962). The chromosomes in 63 of 98 blastocysts

were found to have the male sex chromosome complex to give a ratio of 180 males to 100 females. At birth, however, the ratio was only 106:100 indicating that many more males than females were lost during the gestation period.

The present study gave a sex ratio of 117 males to 100 females on the sixth day of gestation in rabbits. Asdell (1924) found a ratio of 105.4 males to 100 females at birth in rabbits. This also indicates a loss of male embryos during gestation. No difference in the sex ratio was established when the time between gonadotrophin injection and mating was increased. Delayed mating was also reported to have no effect on the sex ratio in the guinea pig (Blandau and Young, 1939) and in the rabbit (Hammond, 1934).

VII. SUMMARY

1. Thirty-six rabbits were mated at various intervals from 0 to 14 hours after an ovulation-inducing injection of 25 I.U. chorionic gonadotrophin. A total of 251 blastocysts were recovered six days later. As the interval between injection and mating increased, the number of blastocysts recovered decreased. The greatest decline in the number of blastocysts recovered occurred when mating was delayed at least 10 hours after gonadotrophin injection.
2. Ten of 80 or 13% of blastocysts from animals mated from six to nine hours after gonadotrophin injection were triploid in chromosome constitution. When karyotyped, seven of the triploids were XXY, and three XXX in sex chromosome complex. No instance of XYY triploids were found.
3. A greater proportion of small blastocysts, which appeared to be retarded in development, were recovered from animals mated from 10 to 14 hours after gonadotrophin injection than at the other delay intervals. Seventeen of 20 blastocysts could be examined chromosomally. One chromosome abnormality, a 43/47 chromosome mosaic, was found.

4. Three chromosome abnormalities were found among 57 blastocysts from animals mated two and four hours after gonadotrophin injection. These were a $2n/4n$ mixoploid and two $43/44$ chromosome mosaics. The incidence of chromosome anomalies at these delay-intervals was 5%.
5. Rabbits mated immediately following an injection of 25 I. U. chorionic gonadotrophin yielded five of 73 or 7% chromosomally abnormal blastocysts. One pentaploid, two mixoploids, a trisomy for chromosome-1 and a blastocyst with 44 chromosomes but an abnormal karyotype were found.
6. When doses of chorionic gonadotrophin varying from 50 to 300 I. U. were administered immediately prior to mating, a decrease in the number of blastocysts recovered occurred with doses of 200 and 300 I. U. Also, two chromosome abnormalities were found in 28 blastocysts examined chromosomally from animals injected with 50 and 75 I. U. gonadotrophin. Both chromosome abnormalities were $43/44$ chromosome mosaics.
7. A control group of eight rabbits received no exogenous gonadotrophin prior to mating. A total of 54 blastocysts were recovered from six of the eight rabbits. Two rabbits did not ovulate, as no corpora lutea were found in the ovaries. One chromosome abnormality, a $44/45$ chromosome mosaic was found among 52 blastocysts examined

chromosomally to give an incidence of 2% chromosome anomalies.

8. The sex chromosome complex was 54% male and 46% female to give a ratio of 117:100 males to females in the 6-day blastocysts. No difference was noted in the sex ratio with delayed mating.

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TABLE I

SYNONYMY OF RABBIT CHROMOSOMES PUBLISHED BY VARIOUS AUTHORS

Nichols et al (1965)	Melander (1956)	Teplitz and Ohno (1963)	McMichael et al (1963)	Pruniéras et al (1965)	Ray and Williams (1966)	Sarkar et al (1962)	Clausen and Syverton (1962)
1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2
3	8	4	4	4 (X?)	4	3	4
4	10	14	13	13	13	5	5
5	17	18	16	16	18	8	9
6	19	19	17	17	19	9	10
7	4	3	3	3	3	10	3
8 (X)	X	9 (?)	X	?	X	X	?
9	9	10	9	9	9	11	6

10	11	11	12	12	12	4	7
11	14	15	14	14*	14	6	18
12	16	16	15	15	15	7	8
13	3	5	5	5*	5	14	15
14	5	6	6	6	6	15	16
15	6	7	7	7	7	16	11
16	7	8	8	8	8	17	12
17	12	12	10	10*	10	12	13
18	13	13	11	11	11	13	14
19	15	17	18	18	16	18	?
20	18	X(?)	19	19	17	19	19
21*	20	20	20	20*	20*	20	?
22	21	21	21	21	21	21	21
Y	Y	Y	Y	Y	Y	Y	Y

85

* Chromosomes with satellites.

TABLE 2

PERCENTAGE OF OVULATIONS (CORPORA LUTEA) RECOVERED
AS 6-DAY BLASTOCYSTS AT VARIOUS DELAY INTERVALS
BETWEEN GONADOTROPHIN INJECTION AND MATING

Delay (hrs.)	No. of Animals	Number of corpora lutea	Number of Blastocysts Recovered	Recovery (%)
0	8*	73	63	86
2	3	36	34	95
4	3	32	28	88
6	3	36	31	86
8	4	49	37	76
9	4	38	24	63
10	2	19	4	21
11	2	23	4	17
12	2	20	4	20
13	3	27	9	33
14	1	10	1	10
	35	363	239	

* Twelve blastocysts recovered from a ninth rabbit are not included in the total for the 0-delay interval, as a count of corpora lutea was not made.

TABLE 3

CYTOGENETIC FEATURES OF CHROMOSOMALLY ABNORMAL 6-DAY BLASTOCYSTS RECOVERED FOLLOWING DELAYED FERTILIZATION

Rabbit	Blastocyst	Delay (Hrs.)	Cells Counted	Diploid and Near Diploid							Polyploid						No. of Karyotypes	Blastocyst Size	Anomaly
				<43	43	44	45	46	47	Exact or near 3n	Exact or near 4n	Exact or near 5n	Exact or near 6n	Exact or near 8n					
1	a	0	30	3	2	2				2	13		2	6	2	very small	Mixoploid ♀		
	b	0	11	3	3	1								4	1	very small	Mixoploid		
2	a	0	23							1	4	18			3	very large	Pentaploid XXXYY		
3	a	0	22	1		4	14	2				1			4	average	Trisomy-1 ♂		
4	a	0	16	1	4	11									5	average	6 acrocentrics ♂		
5	a	2	23	1	3	9	3						7		2	very small	Mixoploid ♀		
6	a	2	30	8	13	9									6	average	43/44 Mosaic		

7	a	4	30	8	10	11	1	7	average	43/44 Mosaic
8	a	6	30				30	4	average	Triploid XXY
	b	6	30				30	4	average	Triploid XXX
	c	6	30				29	3	average	Triploid XXY
9	a	6	22				22	2	average	Triploid XXY
10	a	8	30				26	3	average	Triploid XXY
	b	8	25				24	5	average	Triploid XXY
	c	8	12				12	2	small	Triploid XXX
11	a	8	12				11	3	small	Triploid XXY
12	a	9	13				13	2	average	Triploid XXX
	b	9	17				17	2	average	Triploid XXY
13	a	13	19	2	3	9	1	5	very small	44/47 Mosaic ♀

TABLE 4

THE NUMBER OF 6-DAY BLASTOCYSTS WITH A MALE AND FEMALE SEX CHROMOSOME COMPLEX RECOVERED FROM RABBITS MATED AT VARIOUS INTERVALS AFTER INJECTION OF 25 I.U. CHORIONIC GONADOTROPHIN

Delay (hrs.)	Number of Blastocysts Examined Chromosomally	Sex Chromosome Complex	
		♂	♀
0	* 73	39	33
2	33	21	12
4	24	13	11
6	30	15	15
8	29	18	11
9	21	10	11
10	3	1	2
11	1		1
12	3	2	1
13	9	4	5
14	1		1
Total	227	123	103

* Sex chromosome complex of one mixoploid blastocyst could not be determined.

TABLE 5

NUMBER OF HYPODIPLOID, DIPLOID AND HYPERDIPLOID METAPHASES WITH DELAY
(HRS.) BETWEEN GONADOTROPHIN INJECTION AND MATING

Delay (Hrs.)	Number of Metaphases			Percent of Metaphases		
	Hypodiploid	Diploid	Hyperdiploid	Hypodiploid	Diploid	Hyperdiploid
0	434	873	59	32	64	4
2	248	402	19	37	60	3
4	202	246	19	43	53	4
6	230	412	37	34	61	5
8	150	243	32	35	57	8
9	62	115	8	34	62	4
10-14	120	153	12	42	54	4

TABLE 6

DATA FROM RABBITS RECEIVING VARIOUS DOSES OF CHORIONIC GONADOTROPHIN
IMMEDIATELY PRIOR TO MATING

Dose (I.U.)	No. of Animals	Total No. of Corpora Lutea	Total No. 6-Day Blastocysts	Sex Chromo- some Complex ♂	♀	Abnor- malities No.
0	8	*	54	29	23	1
25	9	*	75	39	33	5
50	1	10	9	3	6	1
75	2	23	23	12	7	1
100	1	8	8	5	3	0
200	1	14	9	4	5	0
300	2	21	7	3	4	0
24			185	95	81	8

* Corpora lutea not counted in all ovaries, of those that were counted 97% and 86% of the ovulations were recovered as 6-day blastocysts at doses of 0 and 25 I.U. HCG, respectively.

TABLE 7

CYTOGENETIC FEATURES OF CHROMOSOMALLY ABNORMAL 6-DAY BLASTOCYSTS RECOVERED FOLLOWING INTRAVENOUS INJECTION OF VARIOUS DOSES OF HCG IMMEDIATELY PRIOR TO MATING

Rabbit	Blastocyst	Dose of HCG (I. U.)	Cells Counted	Diploid and Near Diploid							Polyploid				No. of Karyotypes	Blastocyst Size	Anomaly
				<43	43	44	45	46	47	Exact or near 3n	Exact or near 4n	Exact or near 5n	Exact or near 6n	Exact or near 8n			
1	a	0	34		14	18	1	1							7	average	44/45 Mosaic ♂
2	a	25	30	3	2	2			2		13		2	6	2	very small	Mixoploid ♀
	b	25	11	3	3	1								4	1	very small	Mixoploid
3	a	25	23						1		4	18			3	very large	Pentaploid XXXXY
4	a	25	22	1	4	14	2								4	average	Trisomy-1 ♂
5	a	25	16	1	4	11									5	average	6-acrocentrics
6	a	50	21	4	9	8									4	average	43/44 Mosaic
7	a	75	28	11	10	6	1								7	average	43/44 Mosaic

FIGURE 1

Karyotype from a chromosomally normal 6-day blastocyst. The sex chromosome complex is XY.

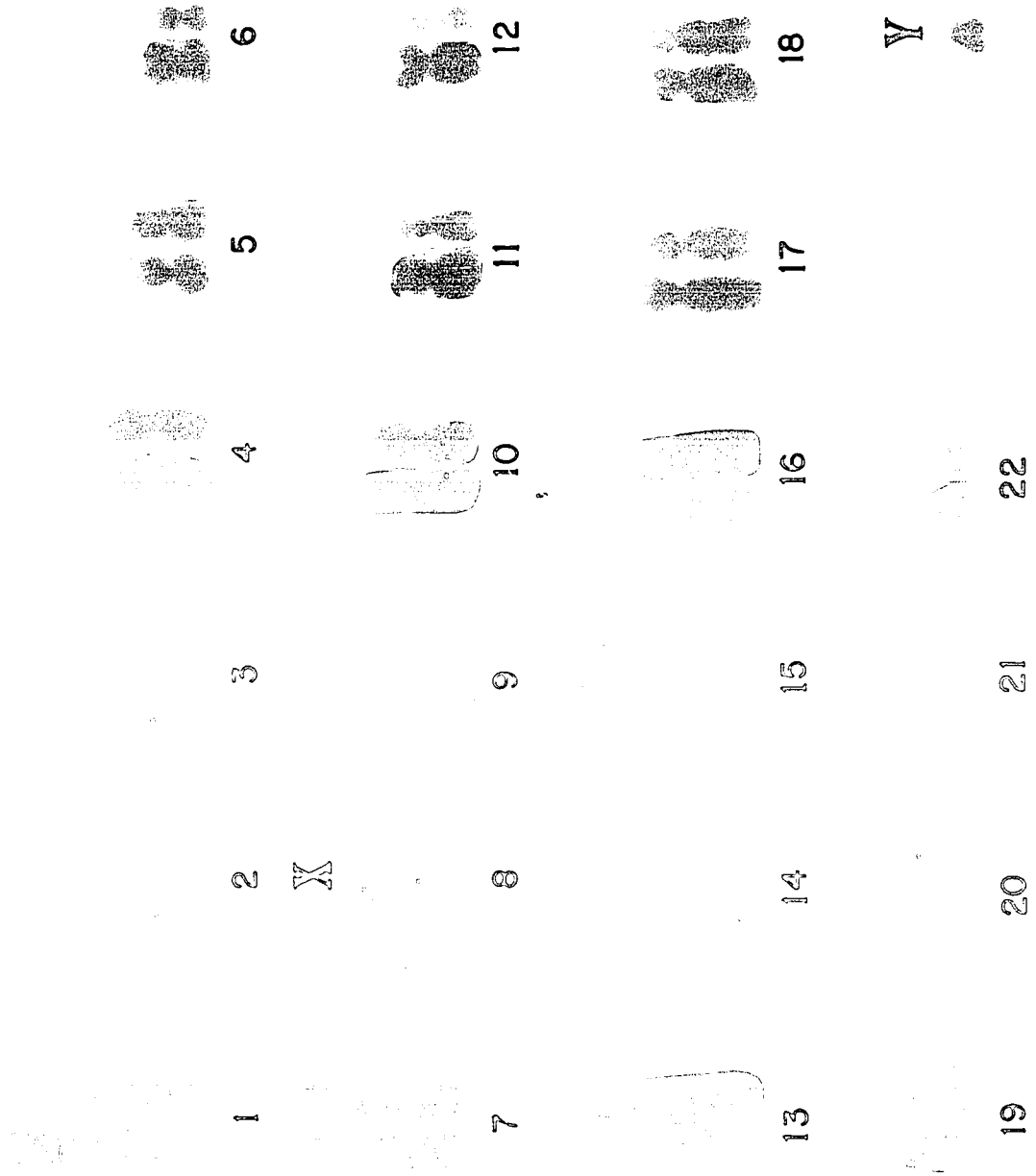


FIGURE 2

Karyotype from a chromosomally normal 6-day blastocyst. The sex chromosome complex is XX.

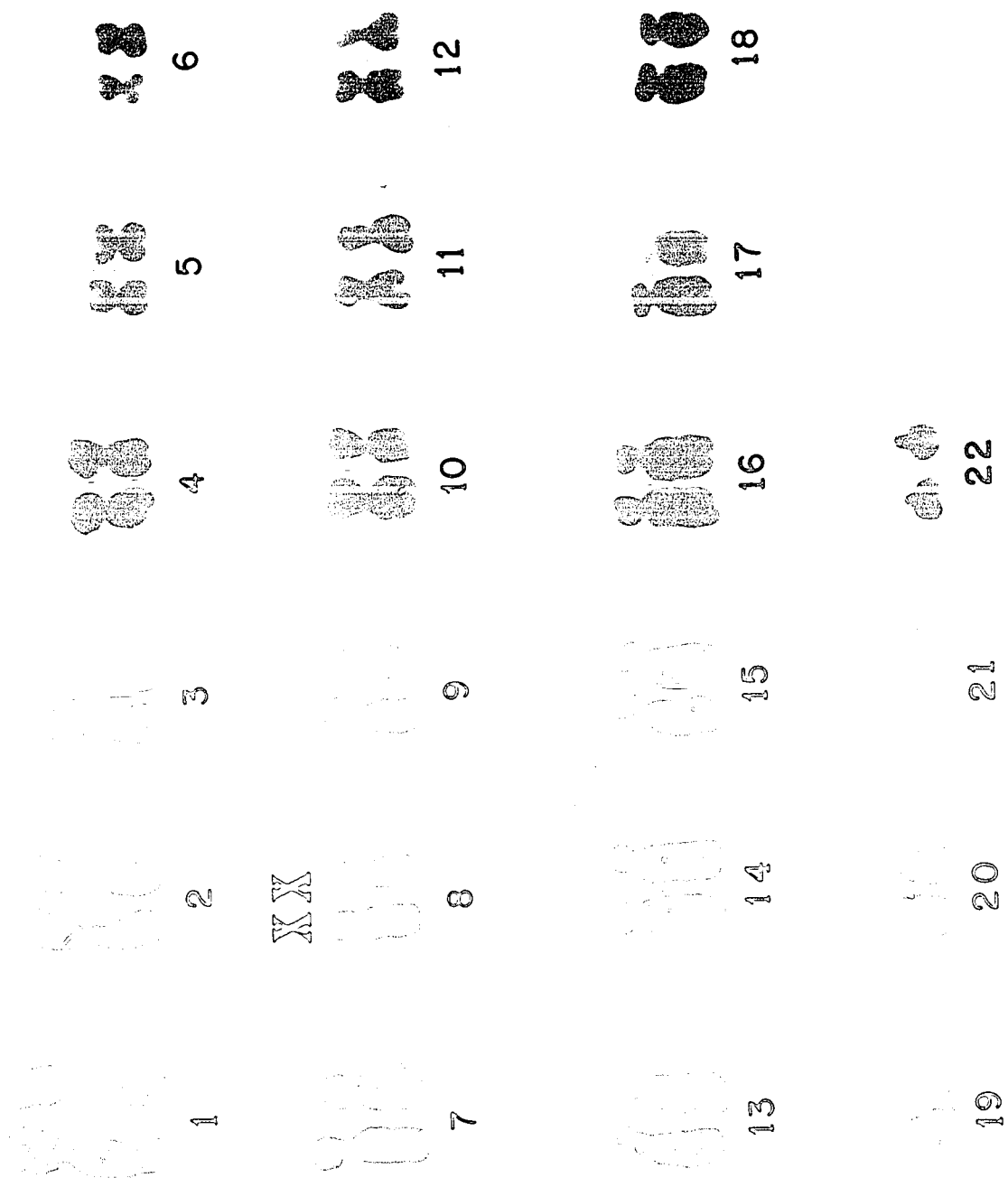


FIGURE 3

Comparison of the number of corpora lutea and the number of 6-day blastocysts recovered with delay between injection of gonadotrophin and mating.

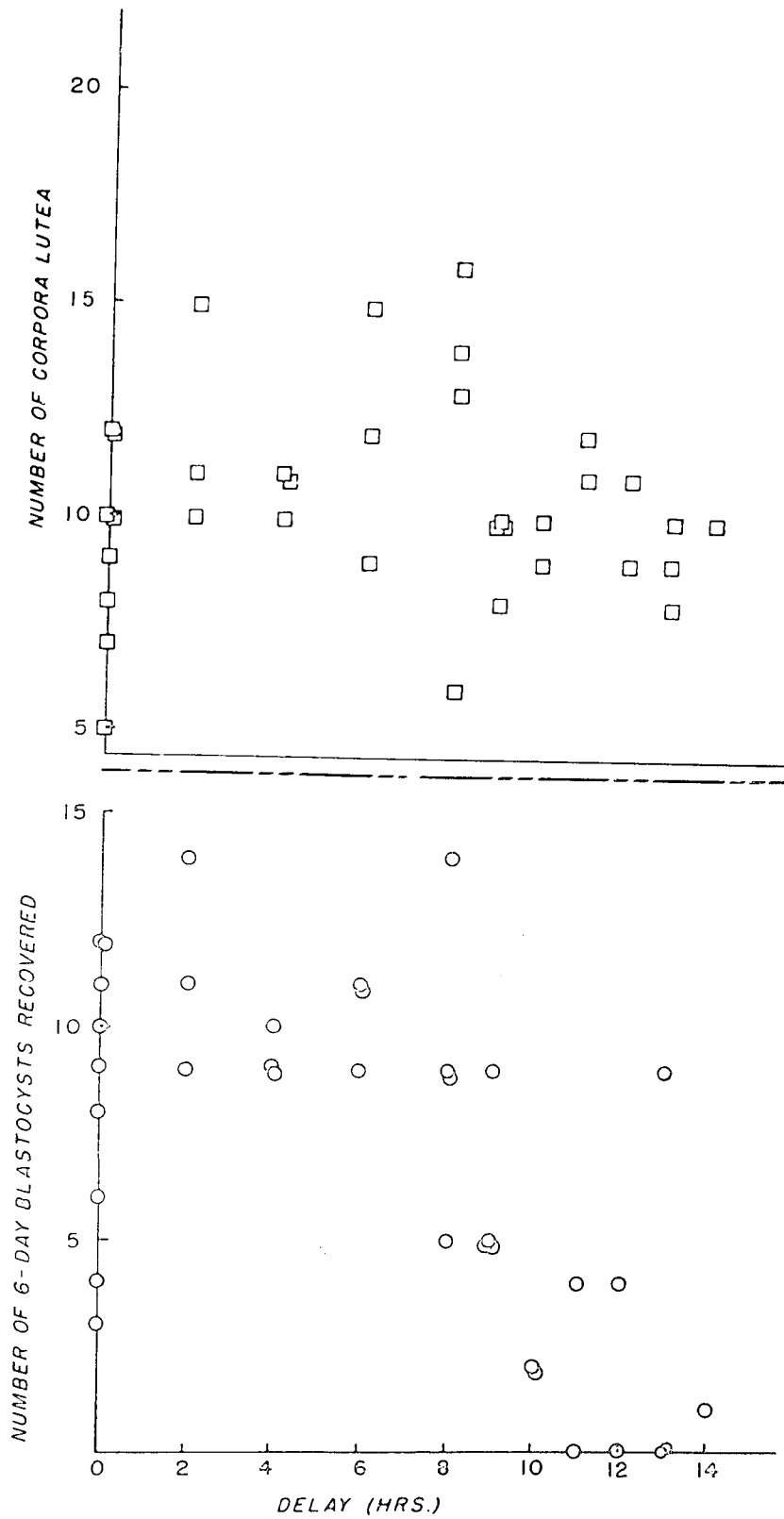


FIGURE 4

Comparison of the recovery of 6-day blastocysts with delay between injection of gonadotrophin and mating.

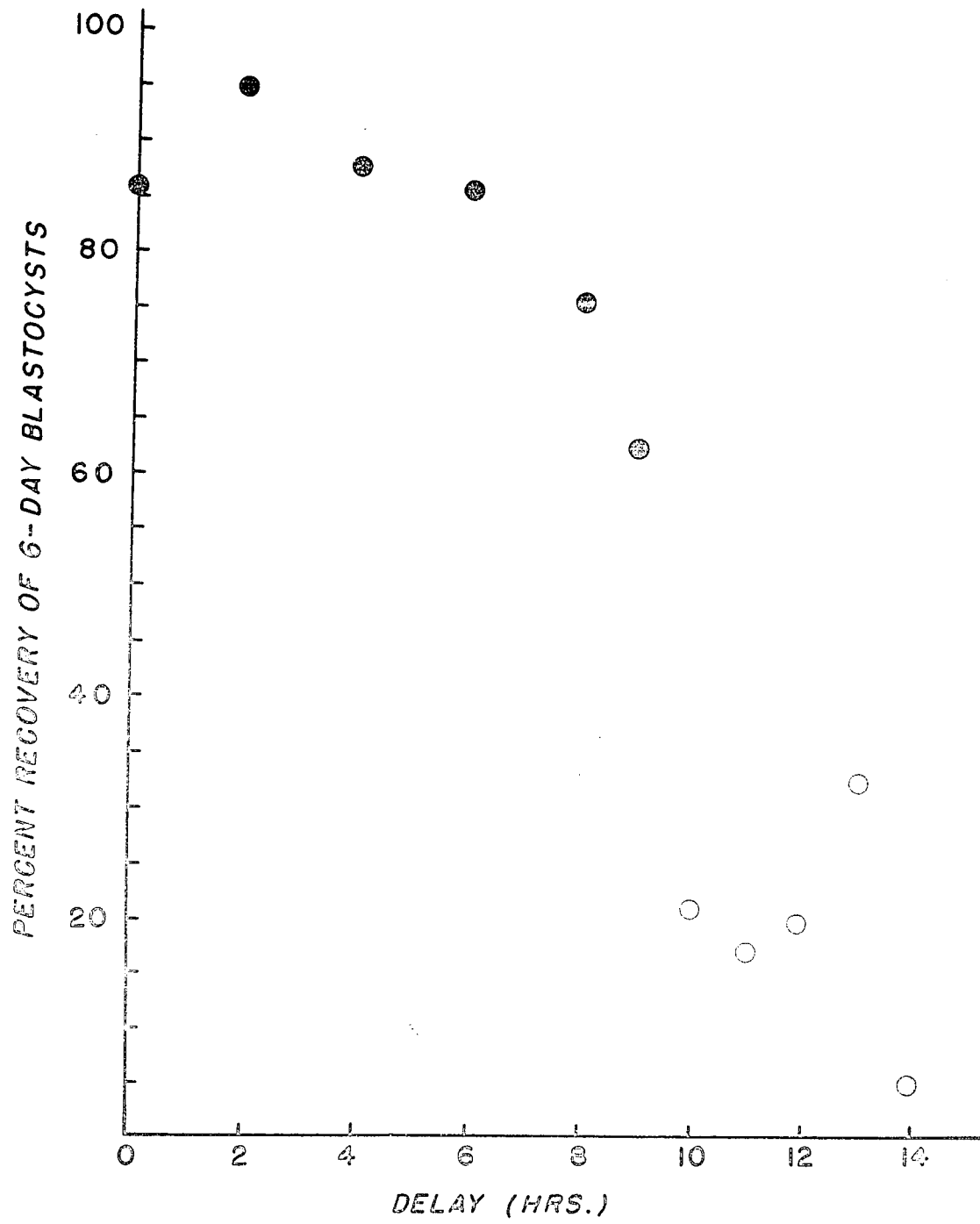


FIGURE 5

Pentaploid karyotype with XXXYY sex chromosome complex.

1 2 3 4 5 6

XXX

7 8 9 10 11 12

13 14 15 16 17 18

YY

19 20 21 22

FIGURE 6

Karyotype showing trisomy for chromosome No. 1.

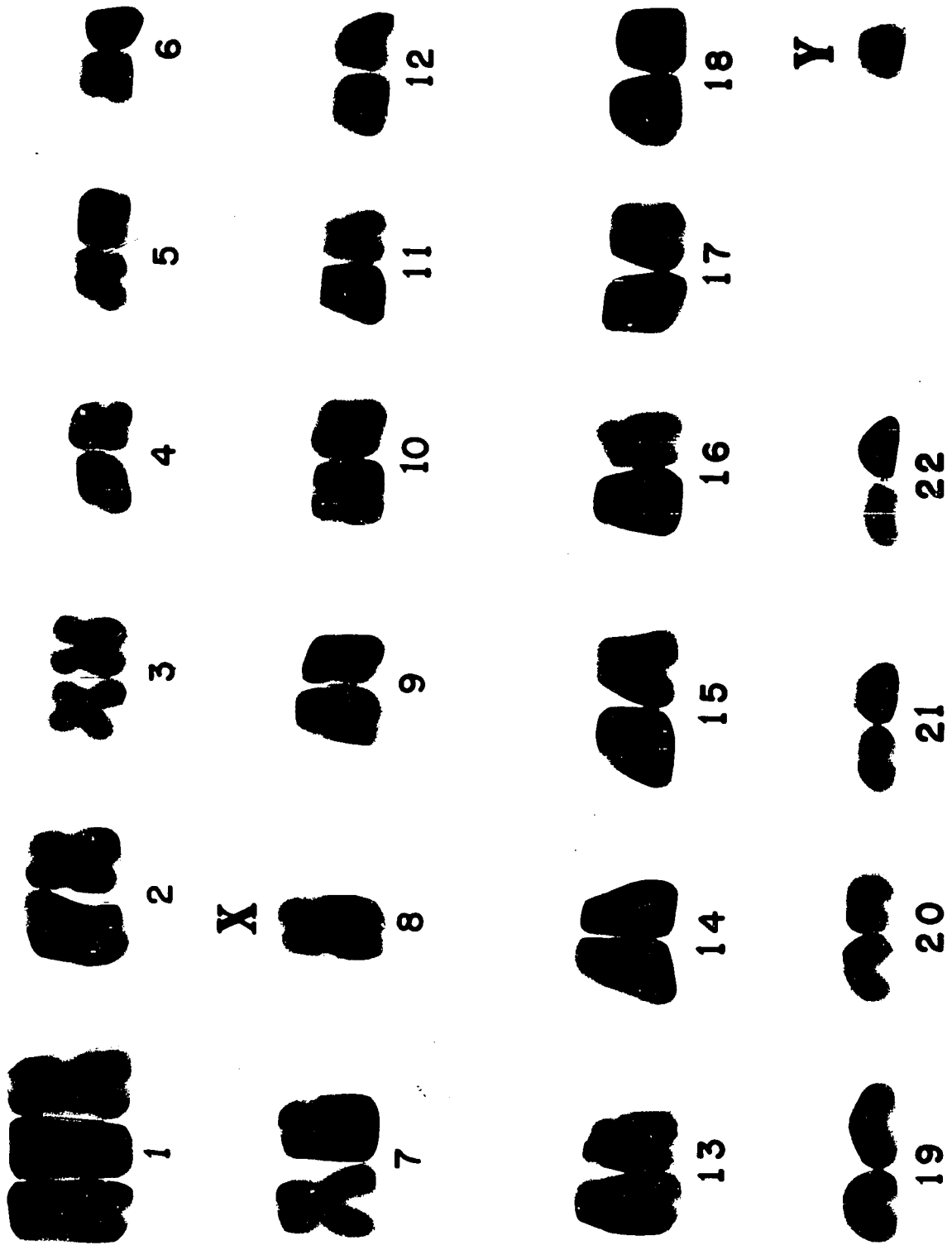


FIGURE 7

**Karyotype from a blastocyst with 6 small acrocentric chromosomes
and a chromosome missing from pair 17.**

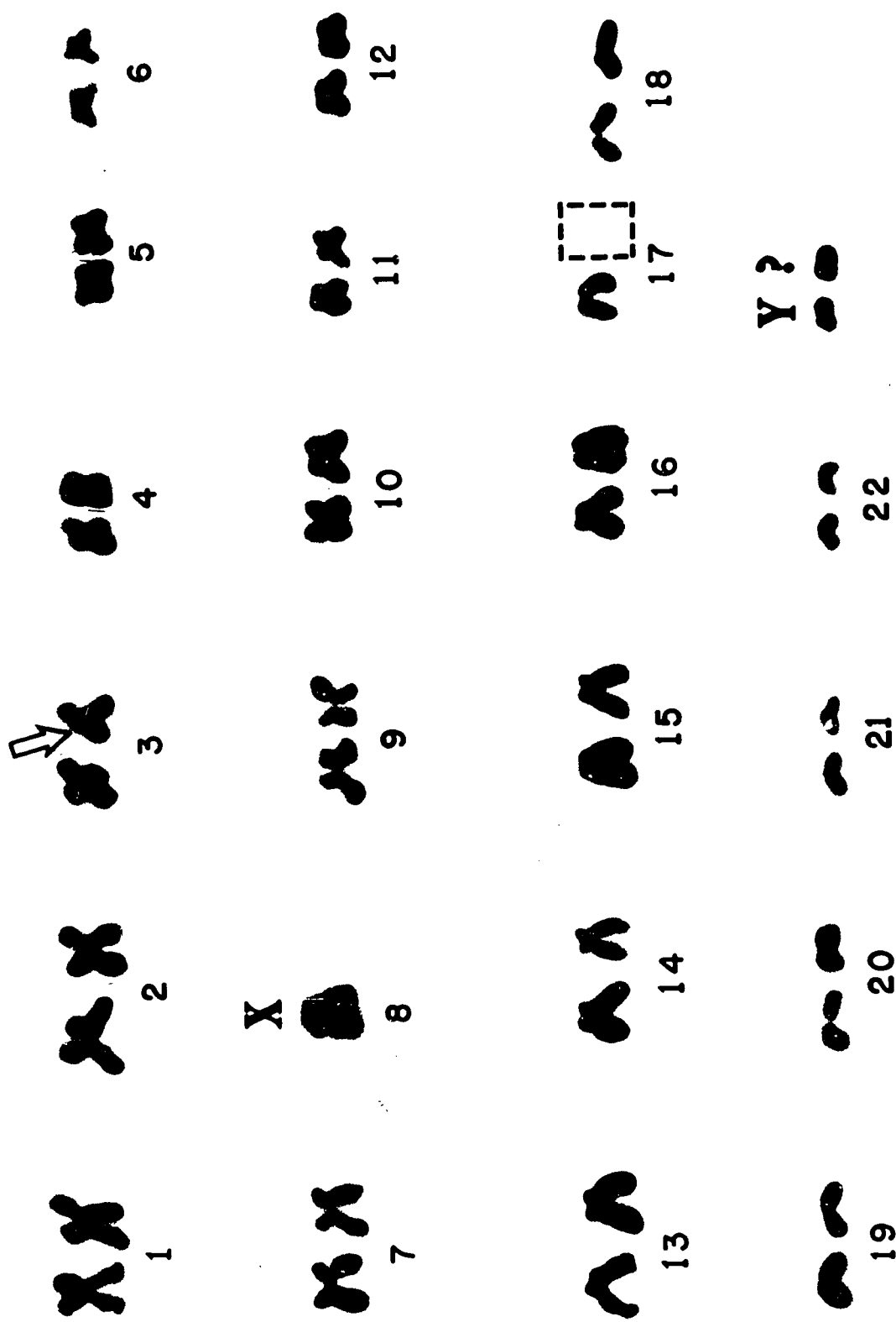


FIGURE 8

Diploid karyotype from 43/44 chromosome mosaic blastocyst from a
2-hour delay-mated rabbit.



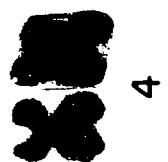
1



2



3



4



5



6

XX



7



8



9



10



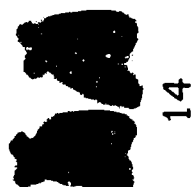
11



12



13



14



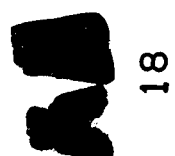
15



16



17



18



19



20



21



22

FIGURE 9

Karyotype from the 43 chromosome population of the 43/44 chromosome mosaic blastocyst from a 2 hr. delay-mated rabbit.

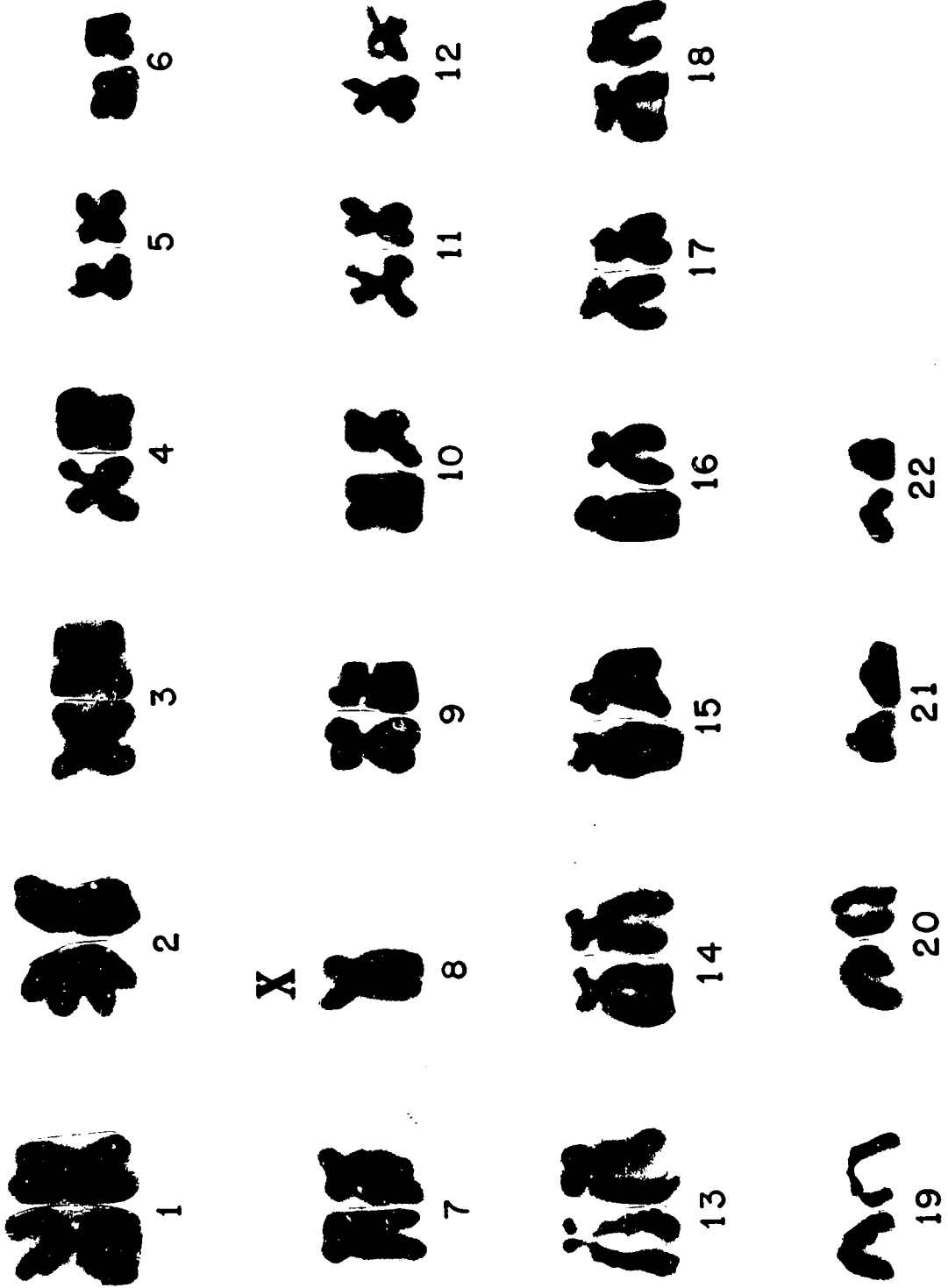


FIGURE 10

Diploid karyotype from a 43/44 chromosome mosaic blastocyst from
a 4 hour delay-mated rabbit.



FIGURE 11

Karyotype from the 43 chromosome population of the 43/44 chromosome mosaic blastocyst from a 4 hour delay-mated rabbit.



X

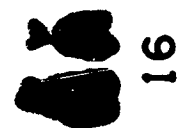
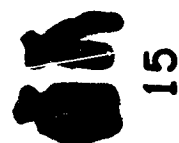
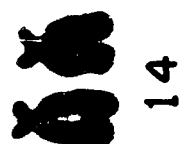
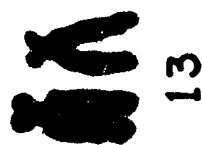
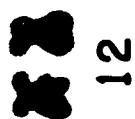
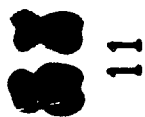
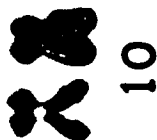


FIGURE 12

Triploid karyotype with XXY sex chromosome complex.

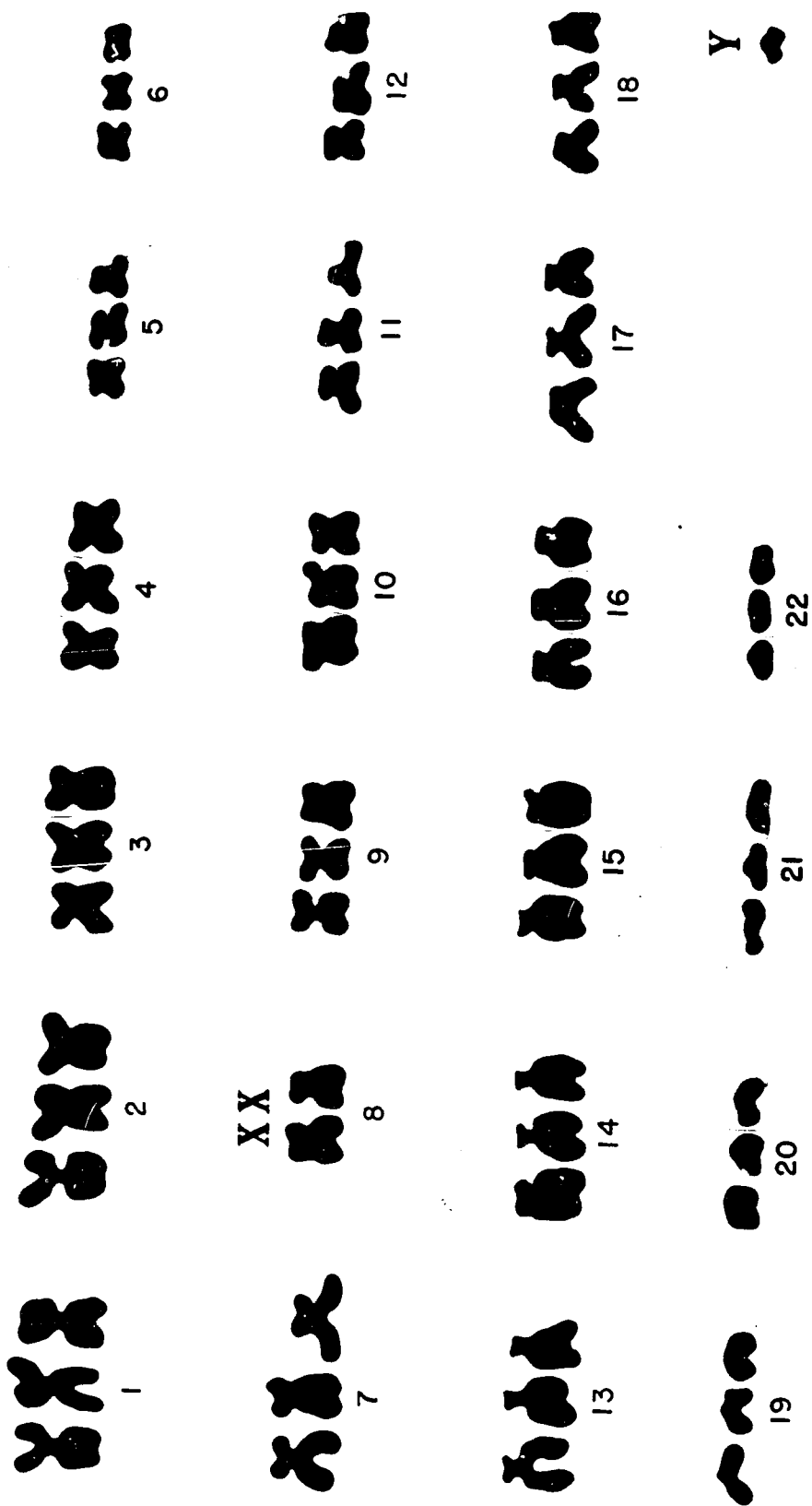


FIGURE 13

Triploid karyotype with XXX sex chromosome complex.

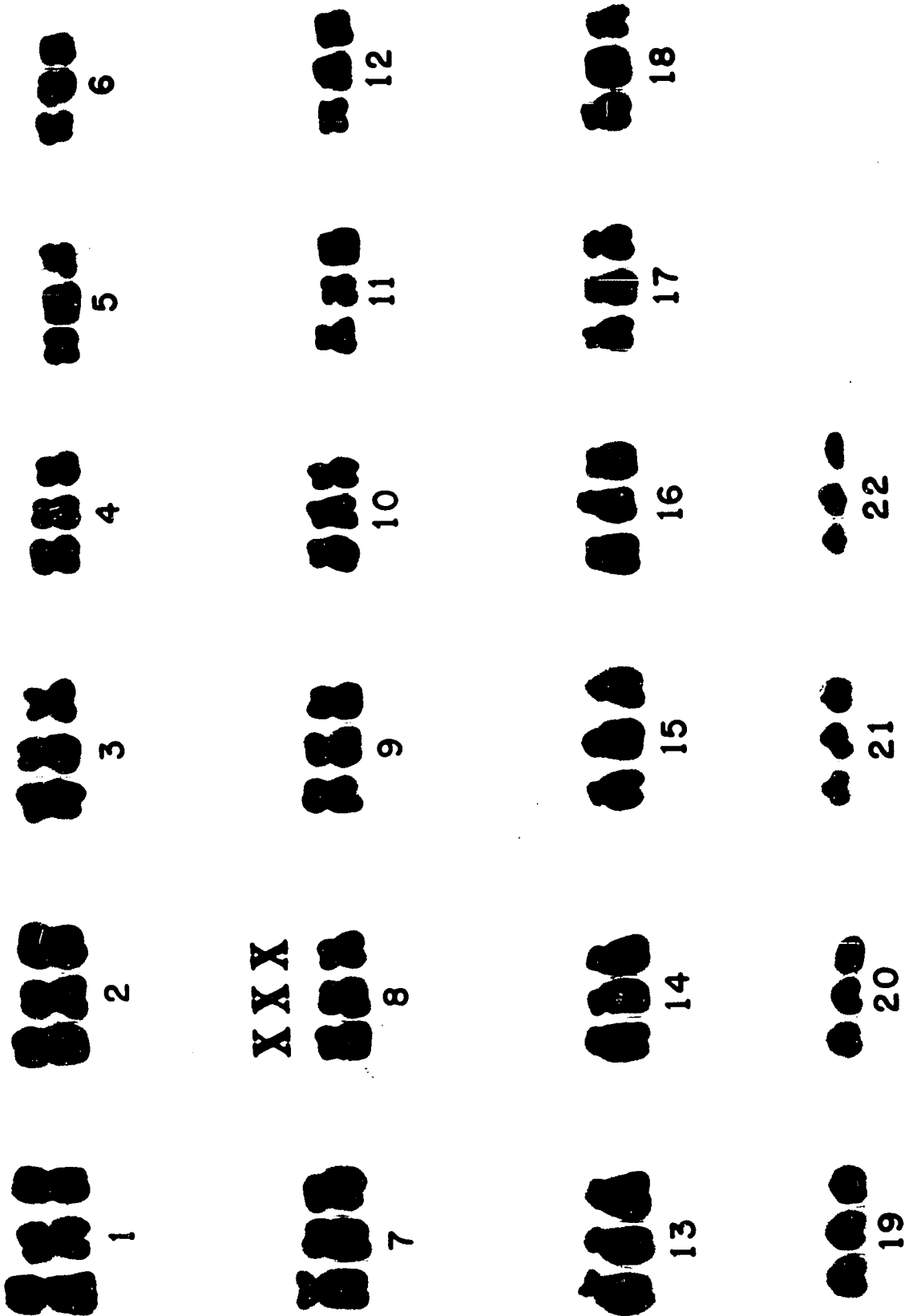


FIGURE 14

Diploid karyotype from 44/47 chromosome mosaic blastocyst from a
13 hour delay-mated rabbit.

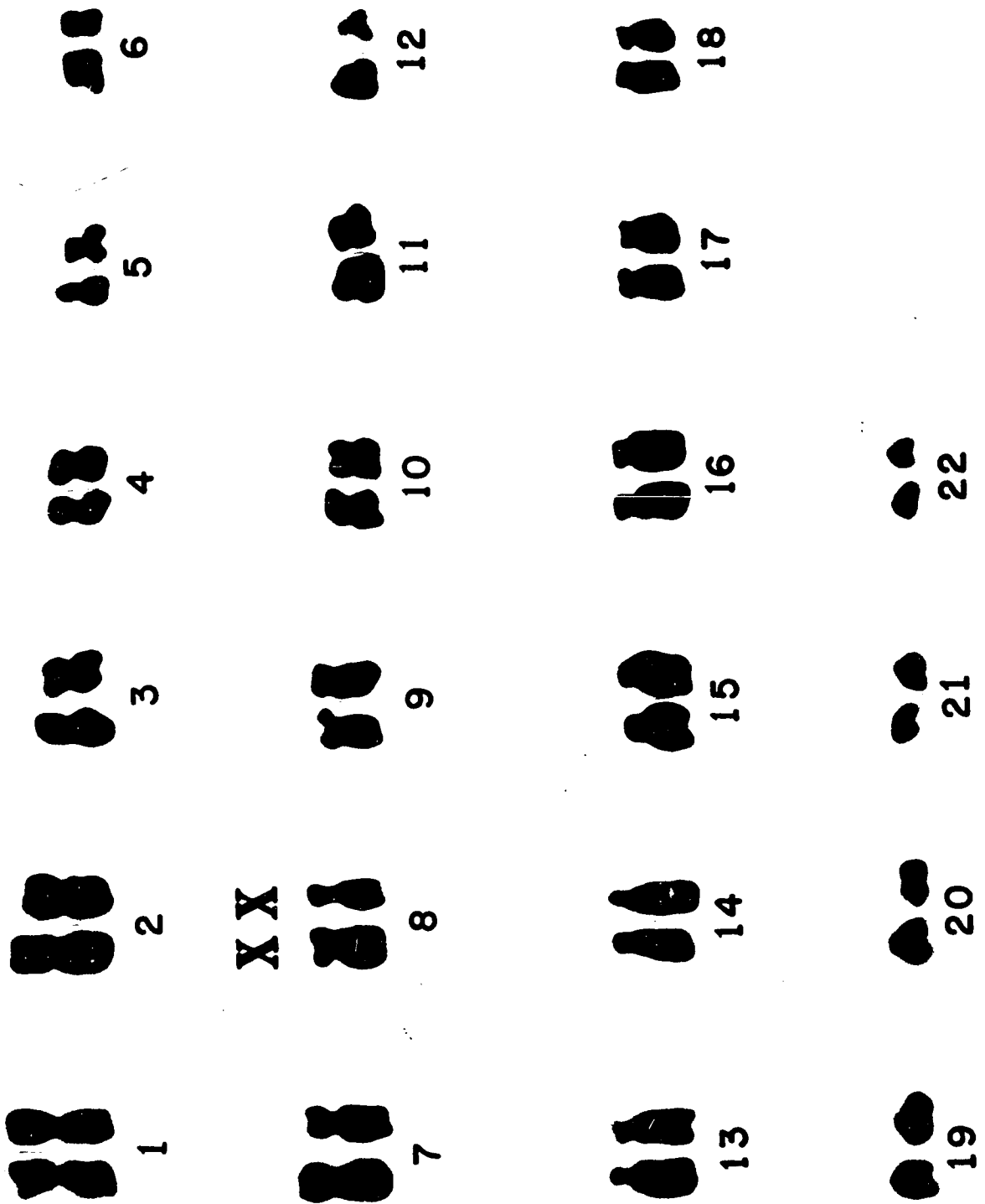


FIGURE 15

Karyotype from the 47 chromosome population of the 44/47 chromosome mosaic blastocyst from a 13 hour delay-mated rabbit.

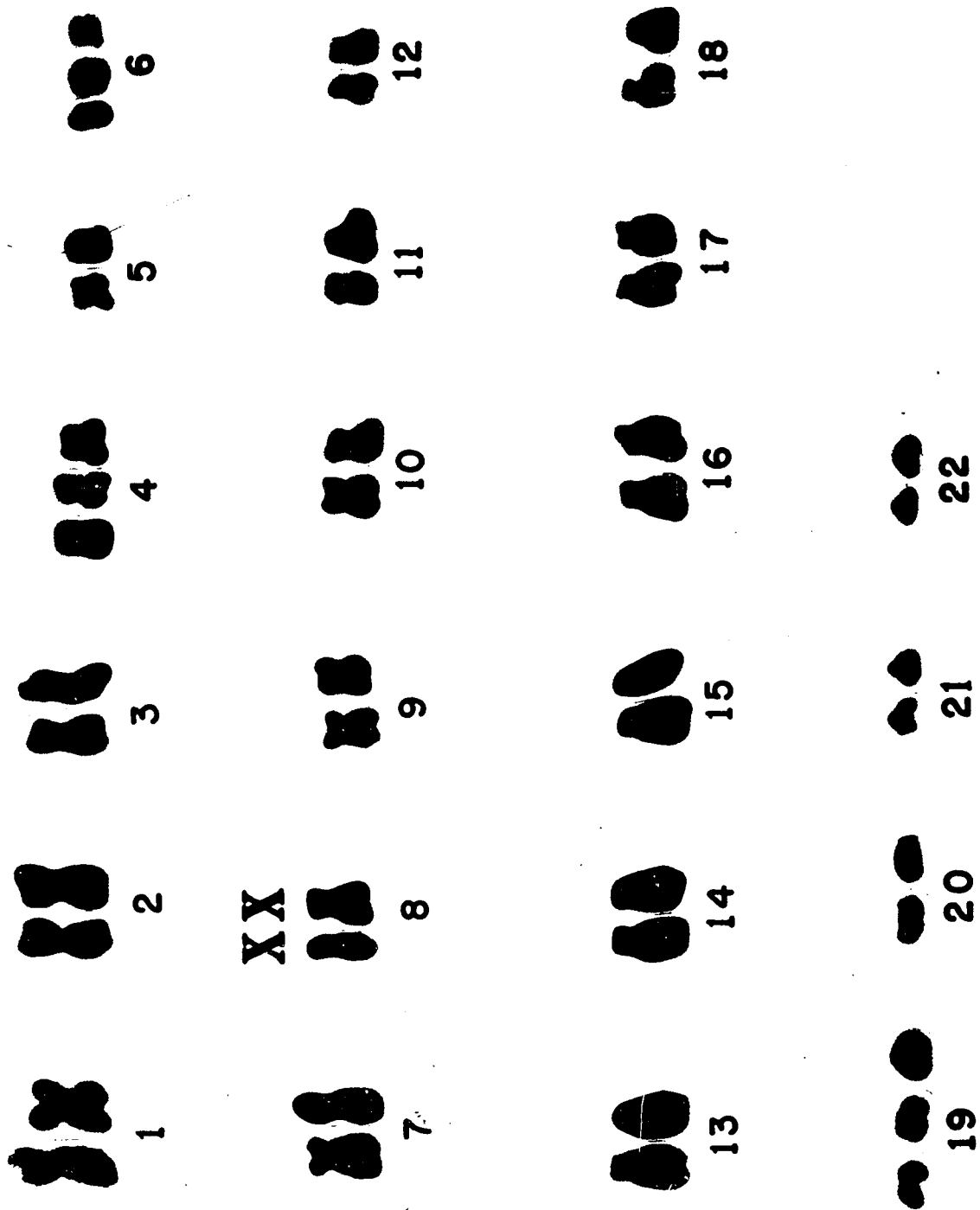


FIGURE 16

Diploid karyotype from a 44/45 chromosome mosaic blastocyst from a rabbit mated without receiving a prior injection of gonadotrophin.

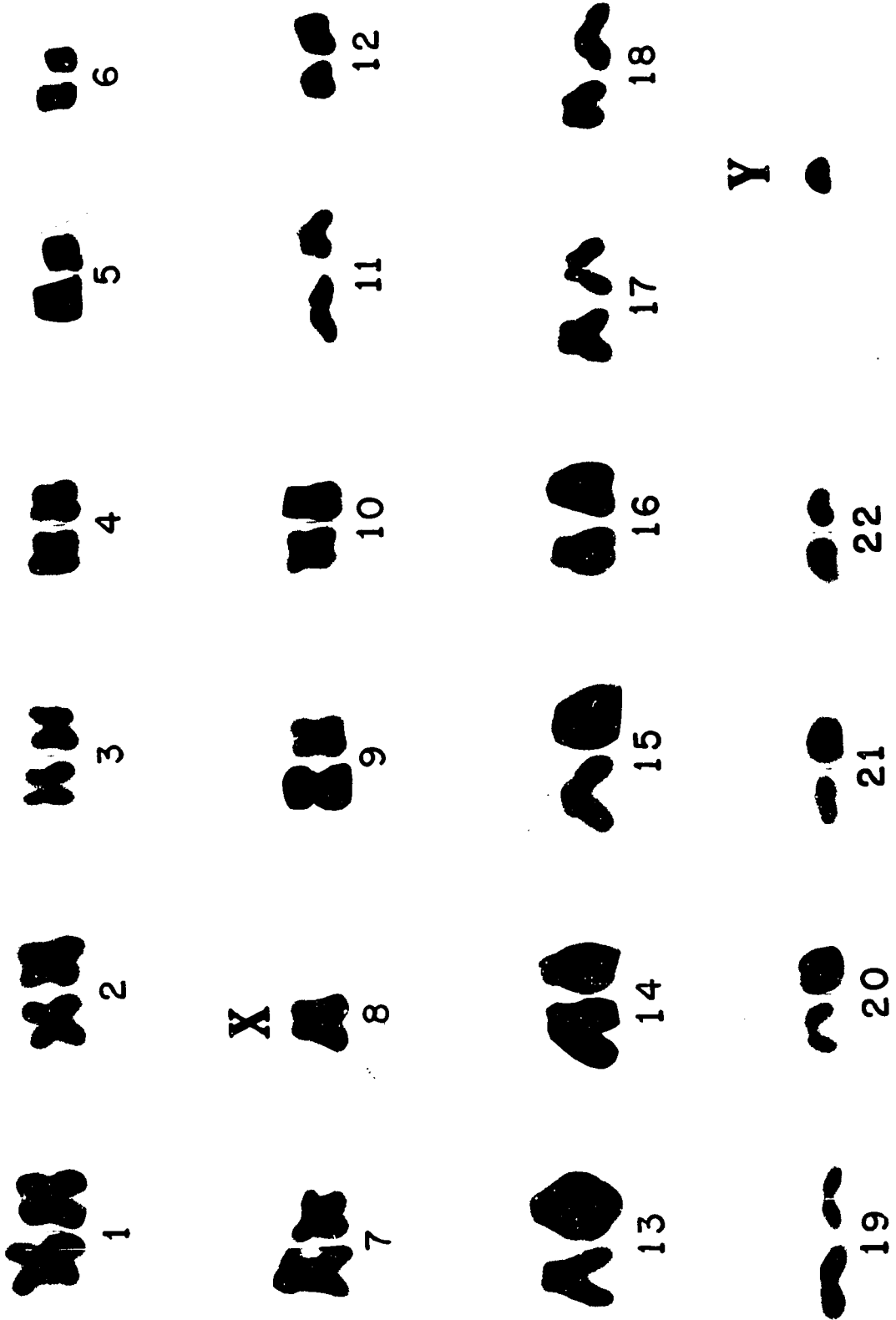


FIGURE 17

Karyotype from the 45 chromosome population of the 44/45 chromosome mosaic blastocyst from a rabbit mated without receiving prior injection of gonadotrophin.

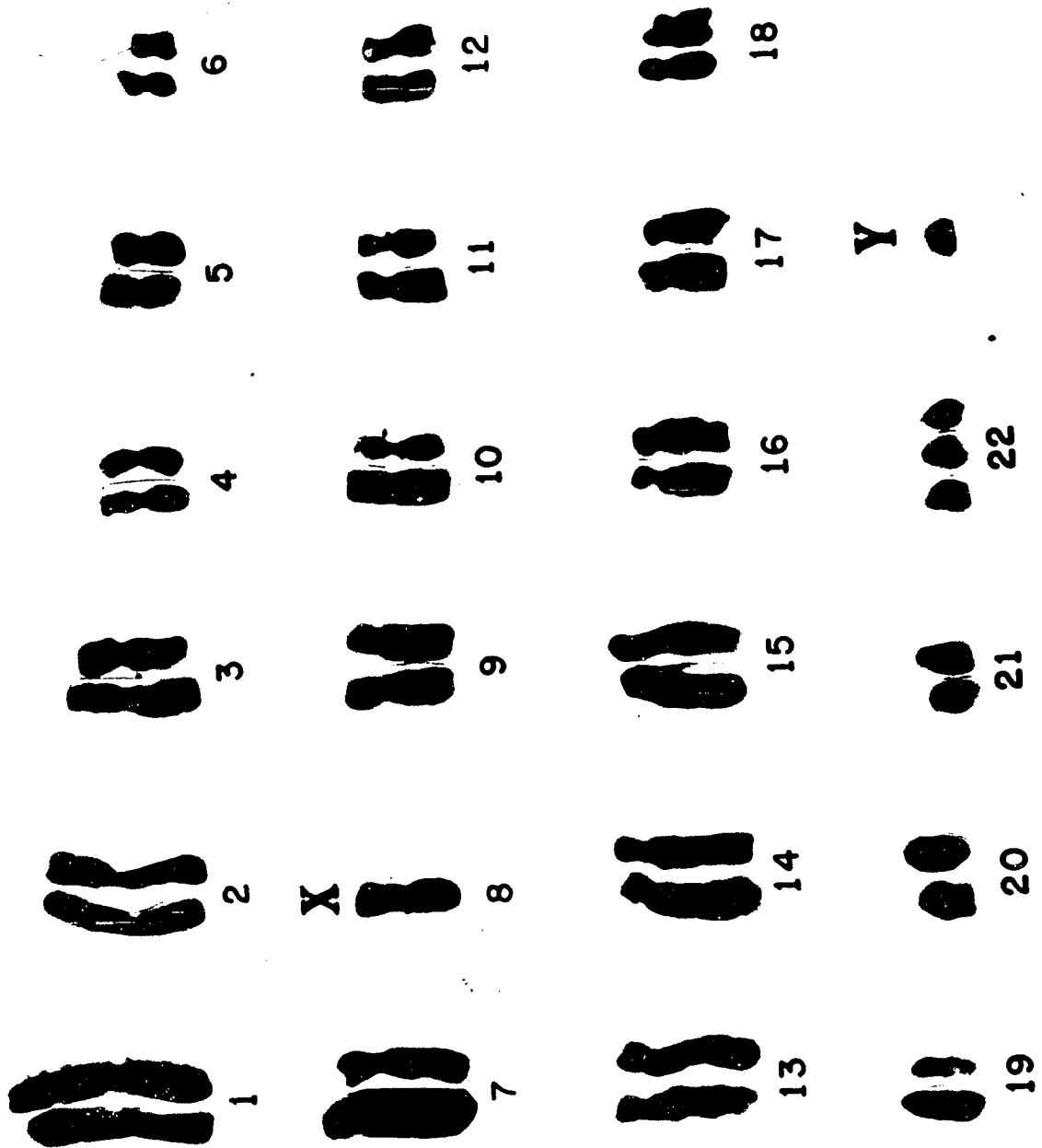


FIGURE 18

Diploid karyotype from a 43/44 chromosome mosaic blastocyst from
a rabbit injected with 50 I.U. HCG prior to mating.

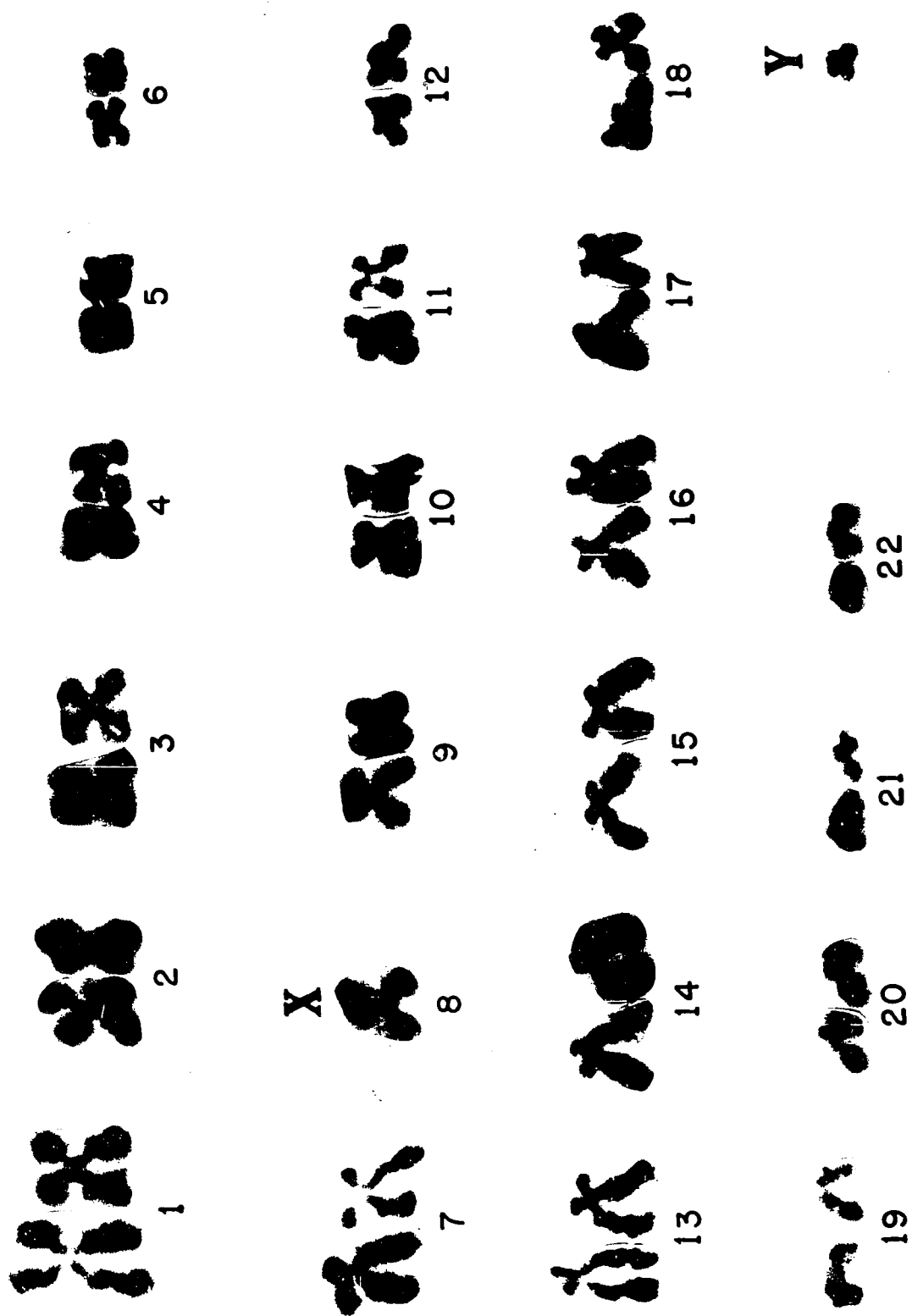


FIGURE 19

Karyotype from the 43 chromosome population of the 43/44 chromosome mosaic blastocyst from a rabbit injected with 50 I.U. HCG prior to mating.

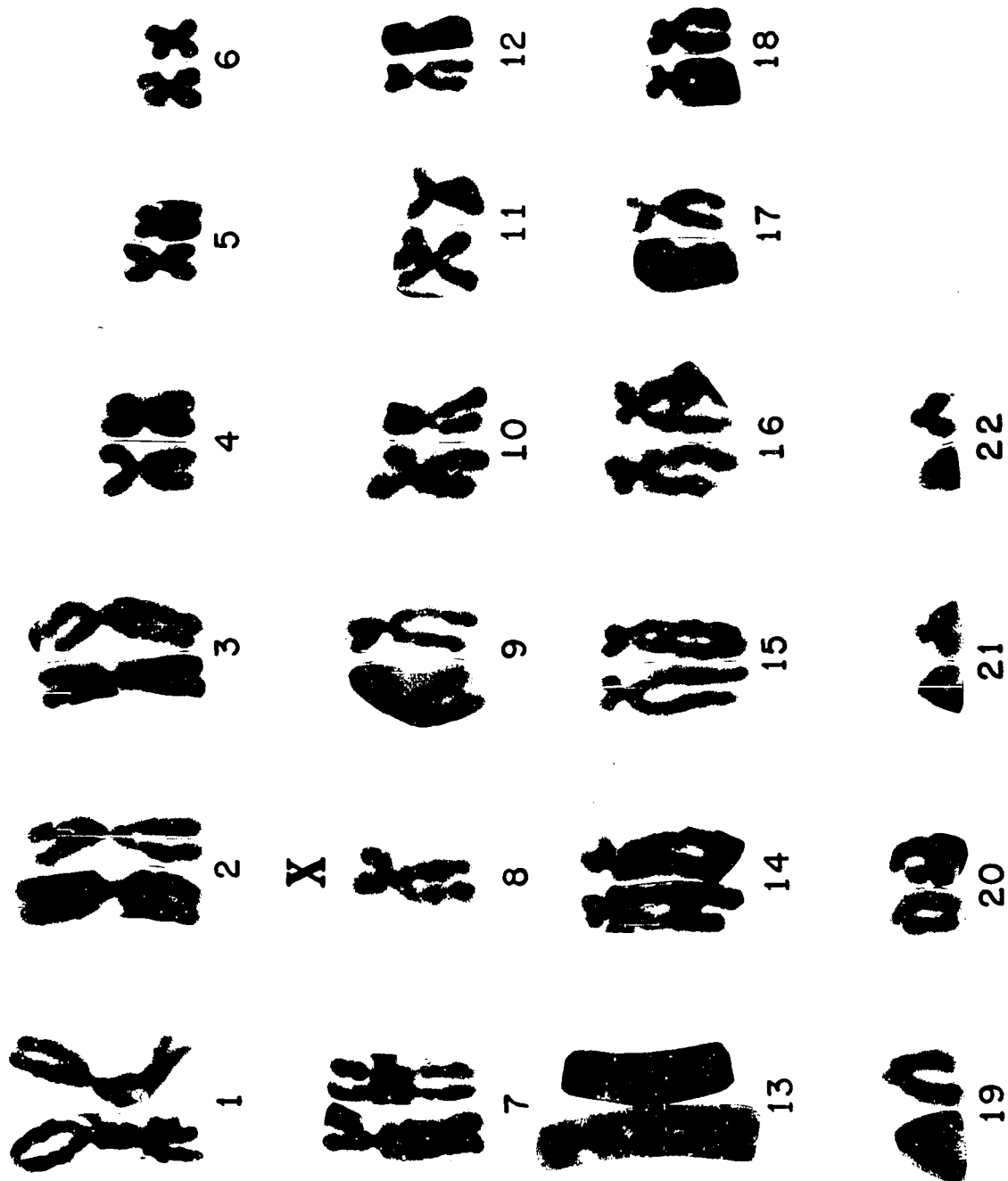


FIGURE 20

**Diploid karyotype from a 43/44 chromosome mosaic blastocyst from
a rabbit injected with 75 I.U. HCG prior to mating.**

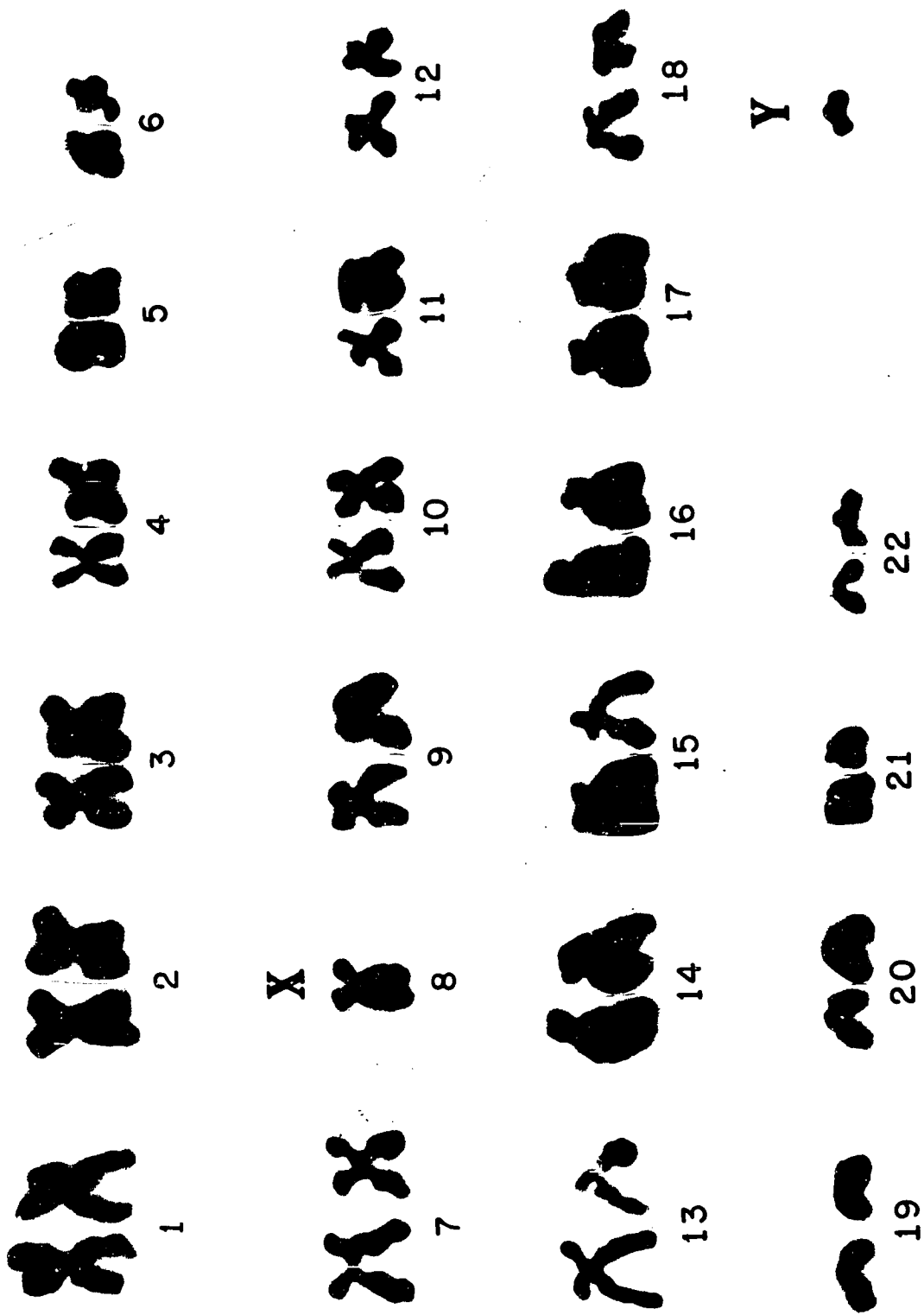


FIGURE 21

Karyotype from the 43 chromosome population of the 43/44 chromosome mosaic blastocyst from a rabbit injected with 75 I. U. HCG prior to mating.

